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Ascorbate Is the Primary Reductant of the Phenoxyl Radical of Etoposide in the Presence of Thiols both in Cell Homogenates and in Model Systems†

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ABSTRACT: Phenoxyl radicals are intermediates in the oxidation of phenolic compounds to quinoid derivatives (quinones, quinone methides), which are known to act as ultimate mutagenic, carcinogenic, and cytotoxic agents by directly interacting with macromolecular targets or by generating toxic reactive oxygen species. One-electron reduction of phenoxyl radicals may reverse oxidative activation of phenolic compounds to quinoids, thus preventing their cytotoxic effects. In the present work, we studied interactions of ascorbate, thiols (glutathione, dihydrolipoic acid, and metallothioneins), and combinations thereof with the phenoxyl radical generated by tyrosinase-catalyzed oxidation of VP-16 [etoposide, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside)], a hindered phenol widely used as an antitumor drug. We found by liquid chromatography–ionspray mass spectrometry and electron spin resonance (ESR) that tyrosinase caused oxidation of VP-16 to its *o*-quinone and aromatized derivative via intermediate formation of the phenoxyl radical. Both ascorbate and thiols (GSH, dihydrolipoic acid, and metallothioneins) were able to directly reduce the VP-16 phenoxyl radical and prevent its oxidation. The characteristic ESR signal of the VP-16 phenoxyl radical was quenched by the reductants. The semidehydroascorbyl radical ESR signal was detected in the presence of ascorbate; thiols did not produce signals in the ESR spectra. In combinations, ascorbate plus GSH and ascorbate plus metallothionein acted independently and additively in reducing the VP-16 phenoxyl radical. Ascorbate was more reactive: the VP-16-dependent oxidation of GSH or metallothionein commenced only after complete oxidation of ascorbate. The semidehydroascorbyl radical ESR signal preceded the quenching of the VP-16 phenoxyl radical by GSH and metallothionein. In the presence of ascorbate plus dihydrolipoic acid, ascorbate was also more reactive toward the VP-16 phenoxyl radical than dihydrolipoic acid, but the ascorbate concentration was maintained at the expense of its regeneration from dehydroascorbate by dihydrolipoic acid. In ESR spectra, the semidehydroascorbyl radical ESR signal was continuously detected and then was abruptly substituted by the VP-16 phenoxyl radical signal. When VP-16 and tyrosinase were incubated in the presence of retina or hepatocyte homogenates, a two-phase lag period was observed by ESR for the appearance of the VP-16 radical signal: an ascorbate-dependent part (semidehydroascorbyl radical observable, sensitive to ascorbate oxidase) and thiol-dependent part (no radical signals in the spectra, sensitive to mersalyl acid). About 50% of the thiol-dependent part of the lag period could be accounted for by endogenous GSH (as revealed by treatment with GSH peroxidase + cumene hydroperoxide). Homogenates prevented VP-16 oxidation by tyrosinase. The ability of ascorbate and thiols, the two major water-soluble intracellular antioxidants, to directly reduce phenoxyl radicals may be an important mechanism of their protective function against cytotoxicity of phenolic/quinoid redox couples.

Oxidation of phenols to quinoid derivatives is recognized to be responsible for the toxicity of this class of compounds (Lewis et al., 1990; Murthy et al., 1990; Thompson et al., 1989). Oxidative metabolic activation of the antitumor drug etoposide [VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside)], as well as oxidation of phenolic metabolites of benzene and the phenolic food preservatives butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are only a few of many examples demonstrating that the general mechanism(s) of the toxicity of phenols may involve oxidation to quinones and/or quinone methides. These quinoid derivatives act as ultimate mutagenic, carcinogenic, and cytotoxic agents by directly interacting with macromolecular targets or by generating toxic reactive oxygen species (Brusick, 1993; Subrahmanyam et al., 1991; Van Maanen et al., 1988). The phenoxyl radical is an unavoidable intermediate in the oxidative metabolism of phenols (Guyton et al., 1991; Kalyanaraman et al., 1989; Nakamura, 1990; Thompson et al., 1989) and either can be oxidized to a quinone (quinone methide) or can undergo one-electron reduction back to the initial phenolic form by an appropriate enzymatic or nonenzymatic reductant (Stoyanovsky et al., 1994).

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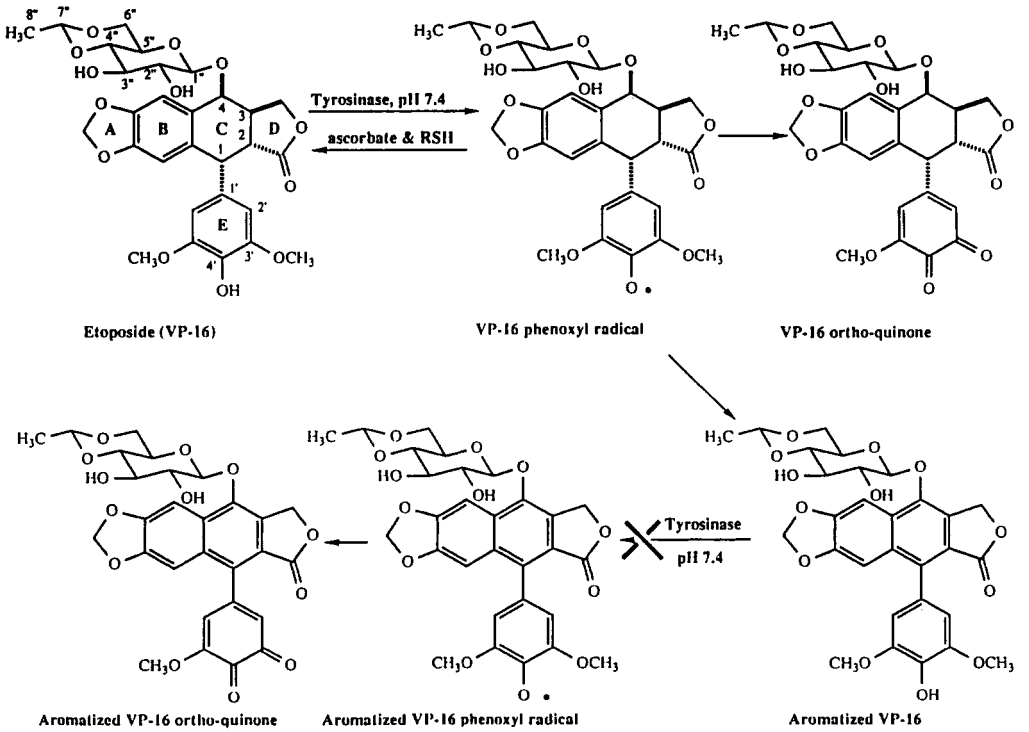

FIGURE 1: Scheme illustrating formation of phenoxyl radicals and quinone derivatives of VP-16 by tyrosinase-catalyzed oxidation and reduction of the VP-16 phenoxyl radical by ascorbate and thiols.

VP-16 is a hindered phenol (Figure 1) widely used as an antitumor drug (Corbett et al., 1993). The presence of the *p*-phenolic group in the E-ring of VP-16 is known to be a structural prerequisite for its antitumor activity (Van Maanen et al., 1988; Loike et al., 1976). The cytotoxicity of VP-16 is considered to be dependent on a dual mechanism of DNA strand cleavage via inhibition of DNA topoisomerase II and/or direct DNA damage (Loike et al., 1976; Long et al., 1984). Oxidative metabolic activation of VP-16 has been suggested to be essential for its cytotoxicity (Van Maanen et al., 1990; Sinha et al., 1985). The phenoxyl radical is the first intermediate in its peroxidative activation (Haim et al., 1986).

Previously, we demonstrated that one-electron reduction of the VP-16 phenoxyl radical prevents its oxidation by tyrosinase both in model systems and in cell homogenates (Stoyanovsky et al., 1994). Ascorbate, reduced glutathione (GSH), and protein thiols are known to be major intracellular reductants (Bergsten et al., 1990; Wells et al., 1990; Chibatsu et al., 1993), which may reduce the VP-16 phenoxyl radical, thus preventing its cytotoxic effects (Stoyanovsky et al., 1994). Elucidation of the hierarchy of interaction of these reductants with the VP-16 phenoxyl radical and with each other may be crucial for elaborating strategies to promote or to suppress VP-16 oxidative metabolism, i.e., to enhance or to attenuate its cytotoxic effects in cancer cells and surrounding tissues, respectively.

In this report we present data on the ability of major intracellular reductants—ascorbate and thiols—to interact with each other and to reduce the phenoxyl radical of VP-16.

MATERIALS AND METHODS

Preparation of Hepatocytes. Freshly isolated hepatocytes were prepared from 200–300-g adult male Sprague-Dawley rats. The cells were isolated from fed animals with collagenase by perfusion (Studer et al., 1982). Immediately after isolation, the cells were layered on a solution consisting of 33% Percoll in 150 mM NaCl and 10 mM HEPES, pH 7.4, and centrifuged for 10 min at 12000*g*. Cell viability was assessed microscopically by trypan blue exclusion and averaged 93%. The isolated cells were incubated in a standard Krebs–Henseleit bicarbonate buffer (KHB). Hepatocyte homogenates were prepared from these cells by sonication (six 5-s pulses at ice-cold temperature) using a tip sonicator (Ultrasonic Homogenizer 4710 Series, Cole-Palmer Instrument Co., Chicago, IL), aliquoted, and frozen at −70 °C. All experiments were carried out as soon as possible after thawing of the aliquots.

Preparation of Retina Homogenates. Male Sprague-Dawley rats were maintained in a weak 12-h cyclic light environment of 4–6 foot-candles/day (lights on 8 am, off 8 pm). Retinas were isolated, homogenized in a glass–Teflon homogenizer, sonicated (six 5-s pulses at ice-cold temperature) using a tip sonicator (Ultrasonic Homogenizer 4710 Series, Cole-Palmer Instrument Co., Chicago, IL), and then aliquoted and frozen at −70 °C. Experiments were carried as soon as possible after thawing of the aliquots.

Preparation of C-Ring Aromatized Derivative of VP-16. The C-ring aromatized derivative of etoposide (AVP-16) was synthesized by the tyrosinase-catalyzed oxidation of VP-16 and subsequent purification of AVP-16 by HPLC. VP-16 (0.8 mM) was incubated for 60 min with tyrosinase (3.7 units/μL) in 0.1 M phosphate buffer (PB) (200 μL, pH 7.4 at 25 °C). The reaction was stopped by adding 200 μL of ethanol and 40 μL of 30% trichloroacetic acid to the incubation medium. After centrifugation of the incubation mixture (5 min at 3000*g*), several aliquots from the resulting supernatant were loaded onto a C_{18} column (Ultrasphere ODS, 5-μm particle size, 4.6 × 250 mm, Beckman). The fractions containing AVP-16 were collected and the resulting solution was saturated with NaCl. The AVP-16 was extracted with $CHCl_3$, and the extracted mixture was dried using Na_2SO_4. The chloroform was subsequently evaporated under a stream of nitrogen at room temperature and the AVP-16 was redissolved in DMSO. HPLC analysis demonstrated the presence of only AVP-16 in the material thus prepared.

ESR Spectral Detection of the VP-16 Phenoxyl Radicals, AVP-16 Phenoxyl Radicals, and Semidehydroascorbyl Radicals. ESR measurements were performed on a JEOL-RE1X spectrometer at 25 °C in gas-permeable 0.8-mm i.d. × 0.013-mm thickness Teflon tubing (Zeus Industrial Products, Raritan, NJ). as previously described (Stoyanovsky et al., 1994). The permeable tube (approximately 8 cm in length) was filled with 60 μL of a mixed sample, folded into quarters, and placed in an open 3.0-mm i.d. ESR quartz tube in such a way that all of the sample was within the effective microwave irradiation area. Spectra were recorded at 335.5 mT center field, 20 mW power, 0.32 G modulation and 2.5 mT/min scan time.

Computer Simulation of ESR Spectra. The ESR spectra were computer-simulated using the following experimental hyperfine coupling constants for the VP-16 phenoxyl radical: $a^{H}OCH_3$ = 0.131 mT (6); a^{H}RING = 0.130 mT (2); $a^{H}\beta$ = 0.40 mT (1); and $a^{H}\gamma$ = 0.06 mT (1); line width 0.02 mT, line shape 60% Lorentzian/40% Gaussian. For the AVP-16 phenoxyl radical: $a^{H}OCH_3$ = 0.139 mT (6); a^{H}RING = 0.139 mT (2); line width 0.02 mT, line shape 80% Lorentzian/20% Gaussian. Computer simulation of the ESR spectra was performed using the software developed in the Laboratory of Molecular Biophysics, NIEHS.

Measurements of the Lag Periods for the Appearance of the VP-16 Phenoxyl Radical Signal in ESR Spectra. Tyrosinase (3–4 units/μL), VP-16 (500 μM), and deferoxamine (100–200 μM) were preincubated in 50 mM phosphate buffer, pH 7.4, for 3 min at 25 °C. All of the above concentrations listed were the final concentrations in the assay system. Tyrosinase was first dissolved in distilled H_2O, while VP-16 was added to the incubation mixture as a DMSO solution. Homogenate (20 μL) was added (time = 0) and the final volume was adjusted to 55 μL with PB. The lag period was then determined as the time prior to the appearance of the VP-16 radical signal (arbitrarily chosen as 2.5× background noise). The lag periods were normalized with respect to protein content in different samples (see below).

Measurements of Lag Periods in the Presence of Exogenously Added Reductants or Oxidants. Ascorbate oxidase (0.004 unit/μL) was preincubated with the homogenate for 1 min and the lag period was determined as above. GSH peroxidase (0.004 unit/μL) and cumene hydroperoxide (cumene-OOH, 500 μM) were preincubated with the homogenate for 20 min and the lag period was determined as above. GSH (25–100 μM) or ascorbate (25–100 μM) was added to the homogenate and the lag periods were compared to lag periods in the model system.

HPLC Assay to VP-16 Oxidation. VP-16 (0.5 mM) and tyrosinase (2.8 units/μL) were incubated in 0.1 M PB, pH 7.4, at 25 °C. Aliquots (10 μL) were taken at given time intervals and transferred from the reaction mixture into 0.3 mL of 50% aqueous CH_3OH. The dispersion thus formed was filtered through a C-18 cartridge (1-mL Sep-Pak cartridge, Waters Division of Millipore, Millipore Co., Milford, MA) and the filtrate was used for HPLC assays. A C-18 reverse-phase column (Ultrasphere ODS, 5-μm particle size, 4.6 × 250 mm, Beckman) was used for HPLC determinations. A Shimadzu LC-10A HPLC system was employed with a LC-600 pump and a SPD-10AV UV detector (detection was by absorbance at 284 nm). The mobile phase was 11:9 CH_3OH–H_2O adjusted to pH 3.1 with CH_3CO_2H. The flow rate was 1 mL/min. Under these conditions the retention time for VP-16 was 6.0 min.

Liquid Chromatography–Mass Spectral Determination of VP-16 Oxidation Products. VP-16 (500 μM) and tyrosinase (3 units/μL) were incubated in 2 mL of 0.1 M PB, pH 7.4, at 25 °C. The mixture was adjusted to pH 1 with HCl and then extracted with either $CHCl_3$ or ethyl acetate (4 × 5 mL). The organic layers were combined and concentrated on a rotary evaporator, and the residue was dissolved in 11:9 CH_3OH–H_2O adjusted to pH 3.1 with CH_3CO_2H. HPLC separations were performed on a Hewlett-Packard 1090 Series II liquid chromatograph/diode array detector using a ODS-Hypersil 5-μm particle size, 100 × 2.1 mm C-18 column (Hewlett-Packard, Palo Alto, CA) at a flow rate of 0.2 mL/min and a mobile phase of 3:2 CH_3OH–H_2O containing 0.1% CH_3CO_2H and 2 mM $NH_4O_2CCH_3$. The diode array detector was set to detect peaks at a primary monitoring wavelength of 280 nm and acquired spectra (190–400 nm) from these peaks every 1.28 s. Pneumatically assisted electrospray (ionspray) mass spectra were obtained on a Perkin-Elmer/Sciex API I mass spectrometer equipped with an atmospheric pressure ionization source and an IonSpray interface. The ionspray interface was maintained at 5 kV and the orifice at 70 V. The curtain gas was high-purity N_2 flowing at 0.6 L/min. High-purity air was used as the nebulizing gas and was maintained at an operating pressure of 40 psi. Analytes were introduced directly to the nebulization/ionization source in the HPLC mobile phase after diode array analysis without splitting of the effluent. Mass spectra were acquired every 6–12 s over mass ranges of 200–800 or 400–700 amu (0.1 m/z resolution). Analytes were detected as their $(M + H)^+$ or $(M + NH_4)^+$ ions.

HPLC Determination of Ascorbic Acid in Tissue and Cell Homogenates. In experiments on reduction of the VP-16 phenoxyl radicals in the presence of tissue and cell homogenates, measurements of ascorbate concentrations were performed by HPLC. After precipitation of proteins in homogenates by 10% CCl_3CO_2H and sedimentation (2000g × 10 min), the supernatant was used for HPLC measurements. We used a ODS-Hypersil 5-μm particle size, 100 × 2.1 mm C-18 column (Hewlett-Packard, Palo Alto, CA) and a mobile phase of 1:24 CH_3OH–H_2O adjusted to pH 3.0 by CH_3CO_2H at a flow rate of 1.0 mL/min. A Shimadzu LC-10A HPLC system was employed. The retention time for ascorbate was 3.4 min. The ascorbate peak was completely eliminated by the addition of ascorbate oxidase to tissue or cell homogenates.

Concentration of ascorbate in model systems was determined by spectrophotometric measurements of its absorbance at 265 nm (ϵ_M = 14.5 mM^{-1} cm^{-1} at pH 7.4) (Buettner et al., 1993) using the Shimadzu 160UV detector interfaced to a Dell 486P/33 personal computer.

Concentration of thiols was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). A standard curve was established by additions of GSH (10–100 μM) to 400 μM DTNB solution in phosphate buffer (10 mM, pH 7.4) and the SH content was calculated (Ellman, 1959). The content of sulfhydryl groups in metallothionein was measured after treatment of the protein with NaCl (3 M). The total protein thiols in homogenates were determined after 30 min of incubation in the presence of 50 mM sodium dodecyl sulfate.

GSH concentration in homogenates was determined by (i) the difference in DTNB-titratable thiols in the presence and in the absence of GSH peroxidase and cumene-OOH or (ii) as the DTNB-titratable thiol groups after precipitation of proteins. Proteins were precipitated with 10% Cl_3CCO_2H

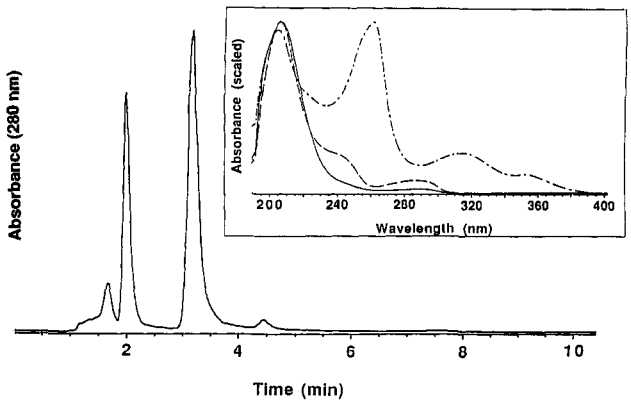

FIGURE 2: HPLC–diode array-detected chromatograms and spectra of products from incubation of VP-16 with tyrosinase. (—) *o*-Quinone of VP-16 (retention time 1.65 min); (- - -) VP-16 (retention time 1.99 min); (- · -) Aromatized VP-16 (retention time 3.19 min). Spectra are normalized to their respective maxima.

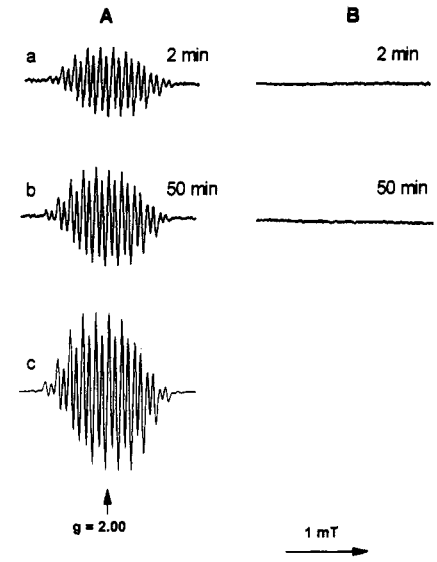

FIGURE 3: (A) ESR spectra of the tyrosinase-induced VP-16 phenoxyl radicals (a, b) and computer simulation of the VP-16 phenoxyl radical ESR spectrum (c). (a, b) Incubation conditions: VP-16 (0.5 mM) and tyrosinase (3.7 unit/μL) in 0.1 M phosphate buffer (pH 7.4 at 25 °C). ESR conditions: center field 335.5 mT, sweep width 1 mT, receiver gain 5×10^2, field modulation 0.32×0.1 mT. (c) The line shape is 60% Lorentzian and 40% Gaussian with a line width of 0.02 mT. (B) ESR recordings obtained from the incubation system containing AVP-16 and tyrosinase. Incubation conditions: AVP-16 (0.3 mM) and tyrosinase (3.7 unit/mL) in 0.1 M phosphate buffer (pH 7.4 at 25 °C). ESR conditions: center field 335.5 mT, sweep width 1 mT, receiver gain 5×10^2, field modulation 0.32×0.1 mT.

and the precipitate was removed by centrifugation sedimentation (2000g × 10 min) at 4 °C. The pH was adjusted back to 7.4 and the concentration of titratable thiol groups in the supernatant was determined with DTNB. The supernatant was also treated with GSH peroxidase and cumene-OOH, after which the thiols were titrated with DTNB.

Depletion of Thiols in Homogenates by Titration with Mersalyl Acid. Mersalyl acid was added to the homogenate in slight excess of DTNB-titratable thiols. Since mersalyl acid inhibited tyrosinase, the excess of mersalyl acid was eliminated by reacting it with GSH and the the excess GSH was removed with GSH peroxidase and cumene-OOH. Thus-treated homogenates were used to determine lag periods in the VP-16/tyrosinase system.

Protein Determination. Protein concentration in the homogenates was determined with the Bio-Rad protein assay kit.

Materials. Acetic acid, ammonium acetate, ascorbic acid, ascorbate oxidase, glutathione (reduced and oxidized), dihydrolipoic acid, sodium phosphates, mersalyl acid, metallothionein, DTNB, GSH peroxidase, cumene-OOH, and tyrosinase were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, $CHCl_3$ or ethyl acetate (all HPLC grade), and dehydroascorbic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). VP-16 was a generous gift from Bristol-Myers Squibb (Syracuse, NY). The protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA).

RESULTS

Mass and UV Spectral Characterization of Tyrosinase-Catalyzed Metabolism of VP-16. Incubation of VP-16 with tyrosinase resulted in the formation of two products as determined by C-18 HPLC/UV/pneumatically assisted electrospray MS. The chromatograms and diode array UV spectra of VP-16 and the two products are shown in Figure 2. Under the conditions used for HPLC/UV/MS determination, VP-16 yielded UV maxima of 206, 242, and 284 nm, a strong m/z 606 $[M + NH_4]^+$ signal, and a weak m/z 589 $[M + H]^+$ signal. The major product was more hydrophobic than the parent compound. Its UV spectrum indicated the presence of a conjugated naphthalene chromophore with maxima at 214, 262, 314, and 352 nm. The m/z 585 $[M + H]^+$ and m/z 602 $[M + NH_4]^+$ ions detected for this later-eluting analyte indicated a loss of 4 mass units from the parent compound. Thus, this compound was determined to be the aromatized C-ring product of oxidation of VP-16 (Figure 1). The UV spectrum of the early-eluting analyte showed maxima at 206, 288, and 368 nm, while its mass spectrum indicated the loss of 16 mass units: m/z 573 $[M + H]^+$, m/z 590 $[M + NH_4]^+$. This oxidation product was determined to be the *o*-quinone of VP-16 (Figure 1). An ion at m/z 592 was also noted for this analyte, which corresponds to the ammoniated adduct of the mass spectrometer-induced reduction product of the *o*-quinone. Both the C-ring aromatized and *o*-quinone derivatives of VP-16 are known to be formed in the enzymatic oxidation of the parent compound, and both are thought to form via competitive disproportionation of the VP-16 phenoxyl radical (Haim et al., 1987). We saw no evidence of products from the aromatized phenoxyl radical, which would include the *o*-quinone of the C-ring aromatized VP-16, by our HPLC/UV/MS analyses. Furthermore, no evidence of this product was noted in ESR experiments (*vide infra*).

ESR Measurements of the Tyrosinase-Induced Radicals in Cell and Tissue Homogenates. Tyrosinase-catalyzed oxidation of the phenolic moiety of VP-16 in the phosphate-buffered aqueous solution (pH 7.4) resulted in the generation of a phenoxyl radical with typical features in the ESR spectrum (Figure 3A) (Kalyanaraman et al., 1989; Mans et al., 1989; Sinha et al., 1990). The oxygen-centered phenoxyl radical exhibits a hyperfine structure similar to that of semiquinone radicals and has the 20-line ESR spectrum which is characterized by the following magnetic resonance parameters: $g = 2.0048 \pm 0.0002$ and hyperfine splitting arising from six identical methoxy protons ($a^{H}OCH3$ = 0.131 mT), two phenoxyl ring protons ($a^{H}RING$ = 0.13 mT), one β-proton (a^{H} = 0.4 mT), and a γ-proton (a^{H} = 0.06 mT). The VP-16 phenoxyl radical was identified by computer simulation of the experimental spectrum using the above hyperfine coupling constants (Figure 3A).

The tyrosinase-induced VP-16 phenoxyl radical ESR signal reached its maximum within 3–5 min of incubation, after

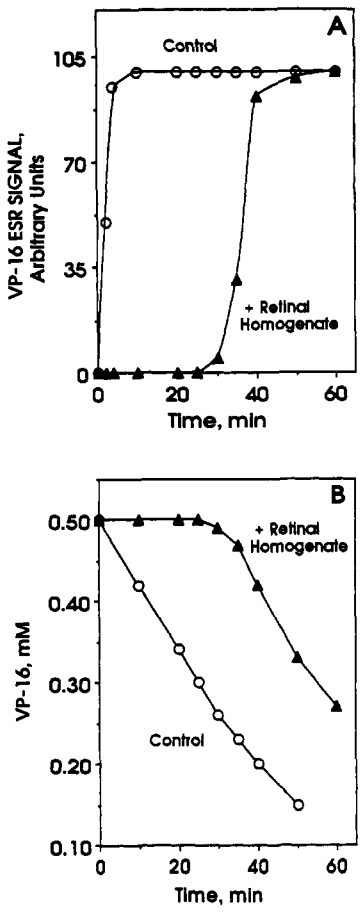

FIGURE 4: Time course of VP-16 phenoxyl radicals (A) as measured by ESR and of VP-16 oxidation (B) the measured by HPLC during tyrosinase-catalyzed VP-16 oxidation in the presence or absence of retinal homogenates. Incubation conditions: Retinal homogenate contained 7.0 mg of protein/mL. Other conditions were as given in the legend of Figure 3.

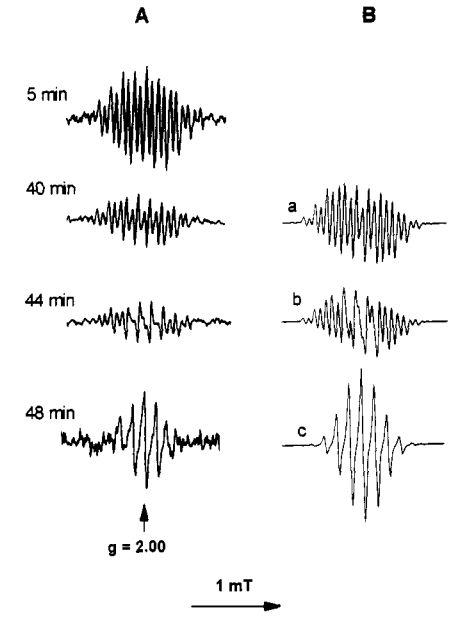

FIGURE 5: (A) ESR spectra of the (horseradish peroxidase + H_2O_2)-induced radicals generated from VP-16. (B) Computer simulation of the AVP-16 phenoxyl radical ESR spectrum and the superposition of VP-16 and AVP-16 ESR signals. (A) Incubation conditions: VP-16 (0.14 mM), horseradish peroxidase (5 units/μL), and H_2O_2 (1.5 mM) in 0.1 M carbonate buffer (pH 9.0 at 25 °C). ESR conditions: center field 335.5 mT, sweep width 1 mT, receiver gain 5×10^2 (1.6×10^3 at 48 min), field modulation 0.32×0.1 mT. (B) The line shape of the AVP-16 phenoxyl radical simulated spectrum is 60% Lorentzian and 40% Guassian with a line width of 0.02 mT (c). Superposition of the simulated ESR spectra of the VP-16 phenoxyl radical and AVP-16 phenoxyl radical: 60%/40% (a), and 40%/60% (b).

which the steady-state concentration of the VP-16 phenoxyl radical did not significantly change over a 40–60-min period and its ESR signal was persistent (its magnitude did not decline over this period of time) (Figure 4). When the tyrosinase inhibitor phenylthiocarbamide (5 mM) (Korn et al., 1988) was added to the incubation medium, the signal of the VP-16 phenoxyl radical immediately disappeared from the ESR spectrum (data not shown), indicating that tyrosinase-catalyzed oxidation of VP-16 produced a steady-state concentration of the VP-16 phenoxyl radical which was high enough to be detected by ESR.

While the formation of the AVP-16 phenoxyl radical has been reported to occur under special conditions (Kalyanaraman et al., 1989), the signal of its radical was not present in the ESR spectra produced by tyrosinase-catalyzed oxidation of VP-16. Even when purified AVP-16 was incubated with tyrosinase, we were not able to detect the signal of its phenoxyl radical for the 50 min of incubation (Figure 3B). However, the AVP-16 phenoxyl radical could be registered when VP-16 was incubated with peroxidase + H_2O_2 at pH 9.0 (Figure 5) (Kalyanaraman et al., 1989). In this system, the VP-16 phenoxyl radical was initially observable in the ESR spectra; the signal was substituted over time by a characteristic and transient signal of the AVP-16 phenoxyl radical. Disappearance of the AVP-16 phenoxyl radical signal from the ESR spectrum was not accompanied by the appearance of any other detectable ESR signal. The AVP-16 phenoxyl radical was identified by computer simulation of the experimental spectrum using the hyperfine coupling constants $a^{H}OCH_3$ = 0.139 mT (6) and a^{H}RING = 0.139 mT (2) (Figure 5).

When cell (hepatocyte) homogenates or tissue (retina) homogenates were added to this standard oxidation system, the VP-16 phenoxyl radical could not be observed initially. Instead, a characteristic ESR signal of the semidehydroascorbyl radical was detected in the spectrum (Figure 6). This signal was transient and disappeared after several minutes. After disappearance of the semidehydroascorbyl radical signal, no ESR signals were discernible in the spectrum for some period of time. The typical ESR signal of the VP-16 phenoxyl radical appeared after this period during subsequent incubation. Thus, the tyrosinase-induced VP-16 phenoxyl radical signal appeared in the ESR spectrum after a lag period which consisted of two parts: (1) with no VP-16 phenoxyl radical ESR signal but with semidehydroascorbyl radical ESR signal present in the spectrum and (2) without any ESR signals in the spectrum. The duration of the lag period was linearly dependent on the concentration of homogenates added to the oxidation system both in terms of the persistence of the ascorbyl radical ESR signal and the duration of the second part of the lag period, during which no ESR signals could be found in the spectra (Figure 6, insets). In the absence of VP-16, the magnitude of the semidehydroascorbyl radical ESR signal was considerably lower than that in the presence of VP-16 and the signal did not disappear during the 50-min incubation period. HPLC determinations demonstrated that the concentration of VP-16 added to cell or tissue homogenates remained unchanged during the lag period before the VP-16 phenoxyl radical was observed (Figure 4A). Appearance of the VP-16 phenoxyl radical signal in the ESR spectra coincided with the onset of its consumption as detected by HPLC (Figure

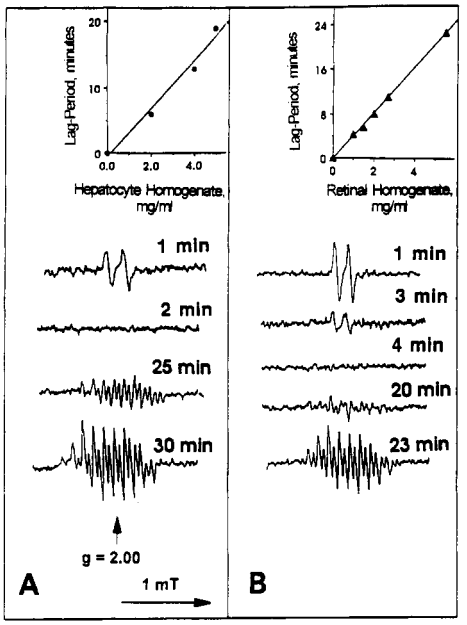

FIGURE 6: ESR spectra of the tyrosinase-induced radicals generated from VP-16 in the presence of tissue and cell homogenates: (**A**) in the presence of retinal homogenates and (**B**) in the presence of hepatocyte homogenates. Incubation conditions: VP-16 (0.5 mM), tyrosinase (3.7 unit/μL), and deferoxamine (250 μM) in 0.1 M phosphate buffer (pH 7.4 at 25 °C) with retinal homogenate (2.0 mg of protein/mL) or hepatocyte homogenate (2.2 mg of protein/mL). ESR conditions: center field 335.5 mT, sweep width 1 mT, gain 1 × 10^3, field modulation 0.32 × 0.1 mT. Insets: Dependence of the lag period for the tyrosinase-induced VP-16 phenoxyl radical ESR signal on the concentration of retinal (A) or hepatocyte (B) homogenates.

4B). In the absence of homogenates, oxidation of the VP-16 proceeded without a lag period.

A 2-min preincubation of the homogenates with ascorbate oxidase abolished that part of the lag period characterized by the presence of the semidehydroascorbyl radical in the ESR spectra. When homogenates were preincubated for 2 min with ascorbate oxidase and then for 5 min with a thiol reagent, mersalyl acid, the VP-16 phenoxyl radical ESR signal appeared in the spectrum immediately (Table 1), indicating that the second part of the lag period, during which no VP-16 radical ESR signal could be detected in the presence of homogenates, was caused by interaction of the VP-16 phenoxyl radical with endogenous thiols. This suggests that ascorbate and thiols are the major contributors to the pool of reductants responsible for the reduction of the VP-16 phenoxyl radical during the lag period. To evaluate the contribution of GSH to the overall interactions of thiols in homogenates with the VP-16 phenoxyl radicals, we treated the homogenates with GSH-peroxidase + cumene-OOH. This resulted in a more than 50% decrease of the lag period, indicating that intracellular GSH was significantly involved in the reduction of the radicals. To investigate the possible interactions of ascorbate and physiologically relevant thiols with the VP-16 phenoxyl radicals, we performed ESR and HPLC studies of tyrosinase-catalyzed VP-16 oxidation in model systems containing ascorbate, glutathione, dihydrolipoic acid, and metallothionein individually and in combinations.

Interaction of Ascorbate and Thiols in Reducing the VP-16 Phenoxyl Radicals in Model Systems: (A) Effects of Individual Reductants on the VP-16 Oxidation. When reductants were added to the incubation system containing VP-16 and tyrosinase, the characteristic ESR signal of the VP-16 phenoxyl radical was not observed (Figure 7). The semidehydroascorbyl radical signal could be immediately recognized in the presence of ascorbate, while no ESR signals could be seen upon addition of reduced thiols (GSH, metallothionein, dihydrolipoic acid) (Figures 8 and 9). In accord with our previous results (Stoyanovsky et al., 1994), the VP-16 phenoxyl radical ESR signal appeared after a lag period, the duration of which was linearly dependent on the concentration of the reductant added (Figure 6, insets).

HPLC measurements confirmed that, in the course of tyrosinase-catalyzed oxidation, the VP-16 concentration did not change during the lag period observed in the ESR measurements, but the oxidative consumption of VP-16 started immediately after the ESR signal of the VP-16 phenoxyl radical was observed (Figures 8 and 9). The reductants (ascorbate, GSH, and dihydrolipoic acid) were continuously consumed during the lag period, and their complete depletion corresponded in time to the onset of the VP-16 oxidation (Figure 8; data for GSH and dihydrolipoic acid not shown). The reaction of sulfhydryl groups of metallothionein with DTNB had two phases: the initial fast reaction was followed by a much slower (an order of magnitude) reaction. This complex kinetics was eliminated by unfolding the protein (treatment with 3 M NaCl). Thus, subsequent measurements of VP-16-dependent SH oxidation in metallothionein were performed after treatment with NaCl. The lag period for VP-16 oxidation in the presence of metallothionein was over before all SH groups of the protein were oxidized (Figure 9).

(B) Effects of Combinations of Reductants on the VP-16 Oxidation. (1) Ascorbate plus GSH. When ascorbate and GSH combined were simultaneously added to the standard oxidation system, the semidehydroascorbyl radical signal was observed in the serial ESR spectra, followed by no detectable signal, then by the VP-16 phenoxyl radical signal (data not shown). The perseverence of the semidehydroascorbyl radical ESR signal was dependent only on the concentration of ascorbate added and was not affected by changes in the GSH concentration. Likewise, the quenching of the ESR signals by GSH was dependent solely on the concentration of GSH and did not respond to changes in the ascorbate concentration.

The time course of oxidation of ascorbate and GSH by tyrosinase plus VP-16 strictly followed the sequence of the signals in the ESR spectra. Initially, only oxidation of ascorbate occurred while the concentrations of GSH and VP-16 remained unchanged (Figure 10). After depletion of ascorbate, the GSH oxidation proceeded. Finally, the VP-16 concentration began to decline after complete consumption of both ascorbate and GSH.

(2) Ascorbate plus Metallothionein. The behavior of the ascorbate/metallothionein couple was similar to that of ascorbate/GSH in terms of both the ESR signals of the radicals generated and the sequence of the oxidations noted by HPLC. The initially observed semidehydroascorbyl radical disappeared and, after a period where no ESR signals were detected in the spectra, was followed by the appearance of the VP-16 phenoxyl radical signal (data not shown). The persistence of the semidehydroascorbyl radical signal was linearly dependent on the ascorbate concentration and did not depend on the metallothionein concentration. The duration of the period when no ESR signal could be observed was determined by the metallothionein concentration independently of the ascorbate concentration.

(3) Ascorbate plus Dihydrolipoate. The combination of ascorbate with dihydrolipoic acid yielded strikingly different results. In the ESR spectra, the semidehydroascorbyl radical ESR signal was persistently registered for a period of time far

Table 1: Effects of Thiol Reagents and Ascorbate Oxidase on the Lag Period and Thiol Content in Retinal and Hepatocyte Homogenates

additions	lag period[a] (min)		thiols (nmol/mg of protein)	
	retinal homogenate	hepatocyte homogenate	retinal homogenate	hepatocyte homogenate
none (control)	20	25	81	88
+ ascorbate oxidase	17	23.5	80	87
+ ascorbate oxidase + GSH-peroxidase + cumene hydroperoxide	8	10	45	48
+ ascorbate oxidase + mersalyl acid	1	<1	10	7

[a] Total lag period was measured in the presence of retinal homogenates (5.0 mg of protein/mL) or hepatocyte homogenates (5.5 mg of protein/mL).

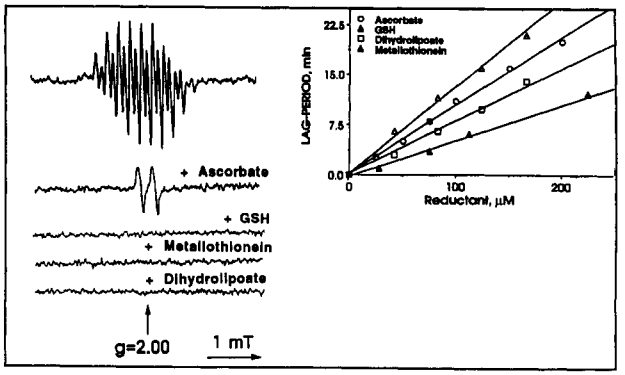

FIGURE 7: ESR spectra of the tyrosinase-induced radicals generated from VP-16 in the presence and in the absence of ascorbate or thiols. Incubation conditions: Ascorbate (100 μM), GSH (100 μM), dihydrolipoic acid (100 μM), and metallothionein (100 μM). All spectra were recorded after 4 min of incubation. Other conditions were as given in the legend of Figure 6. Inset: Dependence of the lag period for the tyrosinase-induced VP-16 phenoxyl radical ESR signal on the concentration of reductants.

exceeding the life-span of the semidehydroascorbyl radical signal obtained in the absence of DHLA and was then abruptly substituted by the VP-16 phenoxyl radical signal (data not shown). There was no part of the lag period during which ESR signals were undetectable (data not shown). The persistence of the semidehydroascorbyl radical signal at a given ascorbate concentration was linearly dependent on the concentration of dihydrolipoic acid added.

In accord with the ESR data, we found that dihydrolipoic acid protected ascorbate against oxidation by the tyrosinase-induced VP-16 phenoxyl radical at the expense of its own oxidation (Figure 11). It is only after the depletion of dihydrolipoic acid that the oxidation of ascorbate commenced. Finally, when both dihydrolipoic acid and ascorbate were consumed, the VP-16 oxidation started. Thus, in contrast to other thiols tested, oxidation of dihydrolipoic acid by the tyrosinase-generated VP-16 phenoxyl radical preceded the oxidation of ascorbate. UV measurements of dehydroascorbate reduction by thiols demonstrated that dihydrolipoic acid rapidly reduced dehydroascorbate to ascorbate, while GSH added at a 10-fold higher concentration caused only very slow formation of ascorbate (Figure 12).

DISCUSSION

Phenoxyl radicals are intermediates in the metabolic activation of different phenols to cytotoxic quinone methides and quinones (Valoti et al., 1989; Shaw et al., 1992; Guyton et al., 1991). Intracellular reductants may prevent the formation of reactive quinones by reduction of phenoxyl radicals, providing a critical protective mechanism against the toxic effects of phenolic compounds. Thus, identification of the intracellular reductants which may be involved in the reduction of the phenoxyl radicals is of great potential import.

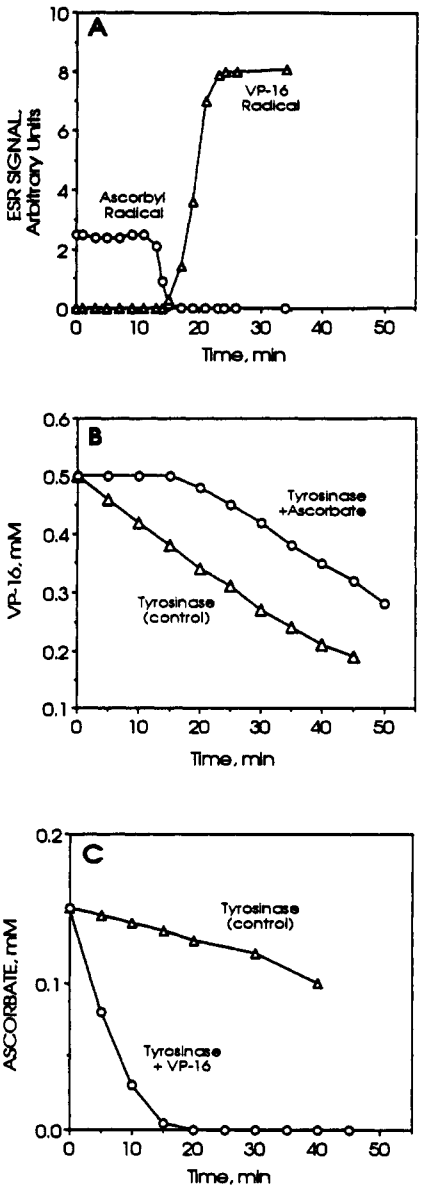

FIGURE 8: Time course of semidehydroascorbyl radicals and of VP-16 phenoxyl radicals as measured by ESR (A) and of VP-16 (B) and ascorbate (C) oxidation as measured by HPLC during tyrosinase-catalyzed VP-16 oxidation in the presence or absence of ascorbate. Ascorbate concentration was 150 μM. Other concentrations were as given in the legend of Figure 6.

VP-16 is a hindered phenol whose metabolic oxidative activation to cytotoxic species is suggested to proceed via the formation of the phenoxyl radical or the semiquinone radical (Kalyanaraman et al., 1989; Haim et al., 1987; Mans et al., 1989; Sinha et al., 1990). The tyrosinase-induced oxidation of VP-16 results in the formation of a well-resolved and characteristic ESR spectrum of the VP-16 phenoxyl radical (Usui et al., 1990; Gantchev et al., 1994; Stoyanovsky et al., 1994), thus providing a convenient system for studies of the

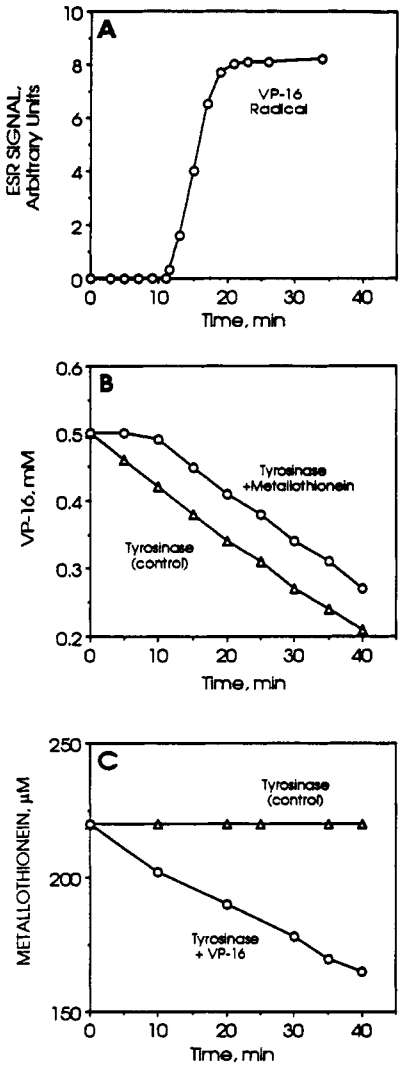

FIGURE 9: Time course of the VP-16 phenoxyl radical ESR signal (A) and of VP-16 (B) and metallothionein (C) oxidation during tyrosinase-catalyzed VP-16 oxidation in the presence or in the absence of metallothionein. Metallothionein concentration was 220 μM. Other conditions were as given in the legend of Figure 6.

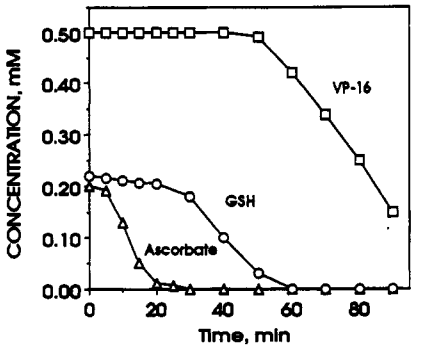

FIGURE 10: Time course of VP-16, GSH, and ascorbate consumption during tyrosinase-catalyzed VP-16 oxidation in the presence of ascorbate plus GSH. Incubation conditions: Ascorbate and GSH concentrations were 200 and 220 μM, respectively. Other conditions were as given in the legend of Figure 6.

reactivity of the VP-16 phenoxyl radical toward physiologically relevant reductants. VP-16 radical reactions examined in this study are limited to those arising from or via the VP-16 phenoxyl radical (Figure 1). The experimental evidence for this is from three sources: (i) ESR spectra, (ii) HPLC/diode array UV chromatograms/spectra, and (iii) LC–MS chromatograms and spectra.

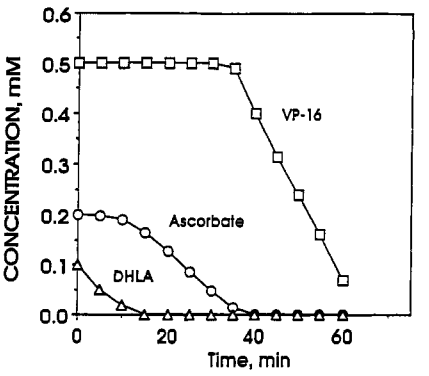

FIGURE 11: Time course of VP-16, dihydrolipoic acid, and ascorbate consumption during tyrosinase-catalyzed VP-16 oxidation in the presence of ascorbate plus dihydrolipoic acid. Incubation conditions: Ascorbate and dihydrolipoic acid concentrations were 220 and 120 μM, respectively. Other conditions were as given in the legend of Figure 6.

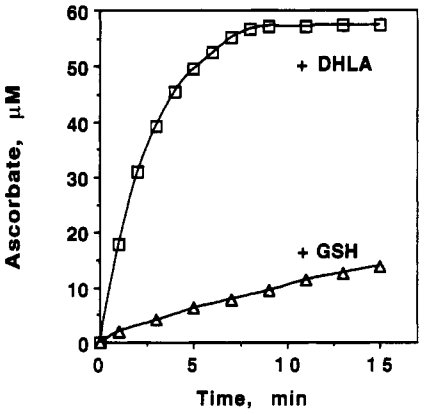

FIGURE 12: Reduction of dehydroascorbate to ascorbate by dihydrolipoic acid and GSH. Incubation conditions: Dihydrolipoic acid (0.1 mM) or GSH (1.0 mM) was added to the solution of dehydroascorbate (100 μM) in 0.1 M phosphate buffer (pH 7.4 at 25 °C) and serial UV spectra were recorded.

We saw no ESR spectral evidence of the phenoxyl radical of the C-ring aromatized form of VP-16 upon incubation of VP-16 with tyrosinase. Moreover, incubation of purified AVP with tyrosinase also did not result in a discernible signal of its phenoxyl radical in the ESR spectrum. However, (peroxidase + H_2O_2)-catalyzed oxidation of VP-16 initially produced VP-16 phenoxyl radicals and then AVP-16 phenoxyl radicals as was reported earlier (Kalyanaraman et al., 1989). Characteristic signals of these radicals could be consequently observed in the ESR spectra. In ESR spectra of the VP-16 phenoxyl radicals, the highest hyperfine coupling constant belongs to Hβ, suggesting high electron density in this region. No electron delocalization involving B- and C-rings is observed after the VP-16 aromatization (Figure 1). This might be most likely due to an unfavorable π–π orbital orientation (orthogonal orientation of the E-ring relative to the B–C-plane). If a resonance structure with an electron delocalized over B–C- and E-rings existed, the resultant radical should most likely have a broad unresolved ESR spectrum at low temperatures (–100 °C), which should be undetectable at room temperatures.

HPLC/diode array UV data and mass spectrometric measurements showed evidence for the formation of only the phenoxyl radical from the parent, reduced C-ring form of VP-16 by tyrosinase, as determined by observation of only oxidized products arising from the phenoxyl radical of VP-16, namely, the *o*-quinone of VP-16 and the C-ring aromatized product (Haim et al., 1987). The *o*-quinone of the aromatized

VP-16, the product of the phenoxyl radical of aromatized VP-16, was not detected.

Our ESR and HPLC measurements indicate that the rates of oxidation of reductants in the presence of tyrosinase were stimulated severalfold by the addition of VP-16, demonstrating that the interaction of reductants with the VP-16 phenoxyl radicals was the majority pathway of their oxidation. However, interactions of the reductants with tyrosinase can be accountable, at least in part, for the duration of lag periods detected in the presence of VP-16.

The time course of ESR signals from radicals generated by tyrosinase-catalyzed oxidation of VP-16 in the presence of cell or tissue homogenates suggests that the VP-16 phenoxyl radical is reduced by different intracellular reductants. The initially observed characteristic doublet ESR signal of the semidehydroascorbyl radical indicates that the reaction of the VP-16 radical with ascorbate takes place:

$$\text{ascorbate} + \text{VP-16}^{\bullet} \rightarrow \text{semidehydroascorbyl radical} + \text{VP-16} \quad (1)$$

This is substantiated by our data showing (i) the elimination of the signal by pretreating homogenates with ascorbic acid oxidase and (ii) the increase of the life span of the semidehydroascorbyl radical ESR signal by adding exogenous ascorbate.

The subsequent quenching of the VP-16 phenoxyl radical ESR signal (during the second part of the lag period when no ESR signals could be observed) suggests that some other reductant(s) were involved in the interaction with the VP-16 radical. Katki et al., (1987) demonstrated that the VP-16 phenoxyl radical is reduced by GSH in organic solvents. We confirmed these data and showed that GSH and DHLA were also able to directly reduce the VP-16 phenoxyl radical in aqueous solutions, in contrast to the vitamin E phenoxyl radical or the acetaminophenol phenoxyl radical, which do not efficiently interact with thiols (Valoti et al., 1989; Kagan et al., 1992). We suggest that reduced thiols are responsible for the part of the total lag period during which no ESR signals were observed in the presence of cell and tissue homogenates added to the standard oxidation system. In accord with this, our experiments with the thiol reagent mersalyl acid demonstrated that the depletion of thiols resulted in a complete elimination of the second part of the lag period.

On the basis of these results we conclude that (i) ascorbate and thiols are the major contributors to the pool of endogenous reductants in hepatocyte and retina homogenates which participate in the reduction of the VP-16 phenoxyl radicals and (ii) ascorbate interacts with the VP-16 phenoxyl radical significantly faster than thiols. While the rate constants for these reactions were not determined in the study, this conclusion can be made on the basis of the sequence of ascorbate and thiol consumption in the course of the tyrosinase-catalyzed VP-16 oxidation. Thus, ascorbate constitutes the primary line of defense against oxidative stress produced by phenoxyl radicals. The administration of VP-16 *in vivo* to mice was shown to decrease the total thiol levels in liver and concomitantly increase the formation of oxidized thiols (Katki et al., 1987). It was suggested that the VP-16 phenoxyl radical was primarily responsible for the thiol oxidation. On the basis of the results of this study, the VP-16 phenoxyl radical-dependent oxidation of thiols could be expected only after depletion of the endogenous pool of ascorbate. An alternative mechanism for thiol consumption may involve their interaction with the VP-16 quinones.

The two important questions which have arisen during our studies are: (i) which thiols are able to directly reduce the VP-16 phenoxyl radicals and (ii) can thiols interact with ascorbate in the course of reduction of the VP-16 phenoxyl radical?

GSH and protein thiols (in particular, metallothioneins) are known to be the major sources of intracellular sulfhydryl groups (Bergsten et al., 1990; Wells et al., 1990, Chibatsu et al., 1993; Chasseaud 1979; Kagi et al., 1988). Both GSH and metallothioneins are abundant in hepatocytes and in the retina (Shan et al., 1989; Tribble et al., 1990; Nishimura et al., 1991). To evaluate the contribution of GSH in the reduction of the VP-16 phenoxyl radicals we used GSH peroxidase and cumene hydroperoxide to specifically deplete endogenous GSH. We found that this pretreatment of homogenates caused 40–45% depletion of total DTNB-titratable thiols and more than a 50% decrease of the duration of the thiol-dependent part of the lag period in ESR measurements. Thus, GSH may be responsible for a significant fraction of sulfhydryl groups participating in the VP-16 phenoxyl radical reduction by homogenates. Its contribution to the intracellular phenoxy radical reduction may be even greater due to continuous regeneration of GSH *in vivo*. Metallothionein was relatively inefficient in the reduction of the VP-16 phenoxyl radical. In fact, only about one of the 20 sulfhydryl groups in metallothionein rapidly reacted with the VP-16 phenoxyl radical in the course of tyrosinase-induced oxidation. This is in agreement with the two-phase kinetics of DTNB titration of thiols in the protein, which was eliminated by denaturing treatment with NaCl. The low efficiency of metallothionein may thus be explained by steric hindrance in the interactions of VP-16 phenoxyl radicals with most of the metallothionein sulfhydryl groups.

Lipoic acid is known to participate in the oxidative decarboxylation of α-keto acids (pyruvate and α-ketoglutarate). Its reduced form, dihydrolipoic acid, is a dithiol with an endogenous concentration which is very low under normal conditions. Recently, pharmacological doses of lipoic acid and dihydrolipoic acid were found to be efficient in therapy of some neural diseases, diabetes, and liver diseases (Kahler et al., 1993; Loginov et al., 1990; Burkart et al., 1993; Ramakirshnan et al., 1992). Thus, dihydrolipoic acid may be an important source of endogenous reductants under these conditions. Our results demonstrate that dihydrolipoic acid can directly reduce the VP-16 phenoxyl radical.

We found that neither GSH nor metallothionein was able to affect the ascorbate-dependent reduction of the VP-16 phenoxyl radical, although both of these thiols were able to reduce the VP-16 radical directly. This suggests that (i) the reactivity of VP-16 radical with ascorbate is by far greater than with thiols and (ii) the reactivity of GSH and metallothioneins with the VP-16 phenoxyl radical is much greater than their reactivity with semidehydroascorbyl radical and/or dehydroascorbate. In contrast, DHLA reduced both the VP-16 phenoxyl radical and dehydroascorbate/semidehydroascorbate. As a result, the ascorbate oxidation preceded the oxidation of GSH or metallothionein but followed the oxidation of DHLA in the course in the tyrosinase-induced VP-16 oxidation. Ascorbate can be efficiently regenerated by DHLA but not by GSH or metallothionein. Thus, ascorbate is the primary reductant involved in the reduction of the VP-16 phenoxyl radicals.

Our results showed that the VP-16 phenoxyl radical can oxidize sulfhydryl groups of both small molecules (GSH, DHLA) and proteins (metallothioneins, other proteins in

hepatocyte and retinal homogenates). This indicates that phenoxyl radicals may exert toxic effects by oxidizing critical thiols of proteins. This mechanism may enhance well-known cytotoxic effects of products of two-electron oxidation of phenols—quinone methides and quinones (Thompson et al., 1989; Mizutani et al., 1991). In particular, interaction of the VP-16 phenoxyl radical with critical thiols in DNA topoisomerase II may be related to this compound's inhibition of the enzyme, which is important for the VP-16-induced tumor cell killing.

It is not known whether the above-described interactions of thiols are specific for the VP-16 phenoxyl radical or may occur with other phenoxyl radicals. For example, we have previously noted that the phenoxyl radical of vitamin E is not efficient in direct interaction with thiols (GSH or DHLA)—the vitamin E phenoxyl radical is not able to react with protein thiols, which may eventually result in oxidative damage (Kagan et al., 1992b). Elucidation of the interaction(s) of the phenoxyl radicals generated from other commonly used phenols (e.g., BHT, BHA, eugenol, acetaminophenol) with protein thiols, however, may be important for understanding the mechanisms of their toxicity. Experiments are now underway to study the reactivity of phenoxyl radicals generated from other hindered phenols (BHT homologues) toward protein thiols relative to the phenols' biological activities.

Ascorbate and GSH are two major water-soluble intracellular antioxidants which act as redox buffers by interacting with reactive oxygen species. The ability of ascorbate and thiols to reduce phenoxyl radicals may be an important mechanism of their protective function against oxidative stress.

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