# Reactions of Phenoxyl Radicals with NADPH-Cytochrome P-450 Oxidoreductase and NADPH: Reduction of the Radicals and Inhibition of the Enzyme†

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ABSTRACT: Phenoxyl radicals are intermediates of one-electron oxidation of phenolic compounds by various peroxidases. This report describes reactions of phenoxyl radicals with human NADPH-cytochrome P-450 oxidoreductase (OR) and NADPH. Purified truncated OR catalyzed quenching of EPR signal of the phenoxyl radical of a vitamin E homolog, 2,2,5,7,8-pentamethyl-6-hydroxychromane. The quenching required both reductase and NADPH and was not supported by NADH. NADPH quenched directly the EPR signal of phenoxyl radical of a phenolic antitumor drug, etoposide, in the absence of the OR. Quenching of the EPR signal was accompanied by increased rate of NADPH oxidation and decreased rate of etoposide oxidation. Phenoxyl radicals of etoposide did not inactivate the OR. In the absence of NADPH, OR was inhibited irreversibly when exposed to phenoxyl radicals of phenol. The activity of the flavoprotein could not be recovered by dithiothreitol (DTT) but the inhibition was prevented by saturation of OR with $NADP^+$ prior to the exposure to phenoxyl radicals. The OR was also inhibited by 5,5′-dithionitrobenzoic acid (DTNB). The inhibition was reversible by subsequent addition of DTT. OR pretreated with DTNB was protected from inhibition by phenoxyl radicals of phenol. The results indicate that phenoxyl radical of 2,2,5,7,8-pentamethyl-6-hydroxychromane is likely reduced enzymatically by transfer of electrons from NADPH via the FAD/FMN of the OR. Phenoxyl radicals with higher redox potential, e.g., phenoxyl radicals of etoposide, oxidize NADPH directly. Phenoxyl radicals of phenol can also inactivate OR likely by oxidation of cysteine 565 in the NADPH binding region of the enzyme.

NADPH-cytochrome P-450 oxidoreductase (OR)[1] (EC 1.6.2.4) is a member of the dehydrogenase electron transfer family of flavoproteins (Massey & Hemmerich, 1980) along with ferredoxin-$NADP^+$ reductase and nitric oxide synthase (Shen & Kasper, 1993). OR transfers hydride ion (i.e., two electrons) from NADPH to FAD, FMN, and finally to the cytochrome P-450 or other electron acceptors such as cytochrome *c*, DPIP, ferricyanide, or menadione (Williams & Kamin, 1962; Vermilion et al., 1981). OR could therefore exert other activities besides its function as an integral part of the cytochrome P-450 enzymic system metabolizing xenobiotics both in the liver and in non-hepatic tissues (Williams, 1976; Hall et al., 1989).

One potential function of OR could be one electron reduction of relatively stable radical intermediates, e.g., radicals of semiquinones and phenolic antioxidants. A free radical reductase activity is associated with various tissues (Fuchs et al., 1990). In general, the free radical reductases require either thiols (Chae et al., 1994; Bast & Haenen, 1990) or NAD(P)H (Villalba et al., 1992; Packer et al., 1989) as donors of reducing equivalents. In some instances, e.g., NADH-ascorbate free radical reductase (Villalba et al., 1992) or thiol-specific antioxidant enzyme (Yim et al., 1994), the specific reductase and its activity were characterized. In other instances, e.g., the α-tocopherol free radical reductase activity of liver microsomes, the responsible enzymes were not identified. The reduction of α-tocopheroxyl radicals by liver microsomes was shown to depend on an enzymatic activity utilizing NADPH (Packer et al., 1989). OR could contribute to this activity of microsomes.

NAD(P)H reduces various phenoxyl radicals with a rate increasing with reduction potential of the phenol/phenoxyl radical couples (Forni & Willson, 1986). The rate of reduction of α-tocopheroxyl radical by NAD(P)H is $<1 \times 10^5$ $M^{-1}$ $s^{-1}$ (Davies et al., 1988). This low rate could be accelerated by enzymes in agreement with the observations

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[1] Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane); DTNB, dithionitrobenzoic acid; DMSO, dimethyl sulfoxide; DOPC, dioleoyl phosphatidylcholine; DPIP, 2,6-dichloroindophenol; DTT, dithiothreitol; EPR, electron paramagnetic resonance; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase; OR, human NADPH-cytochrome P-450 oxidoreductase; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychromane; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; etoposide, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene β-D-glucopyranoside).

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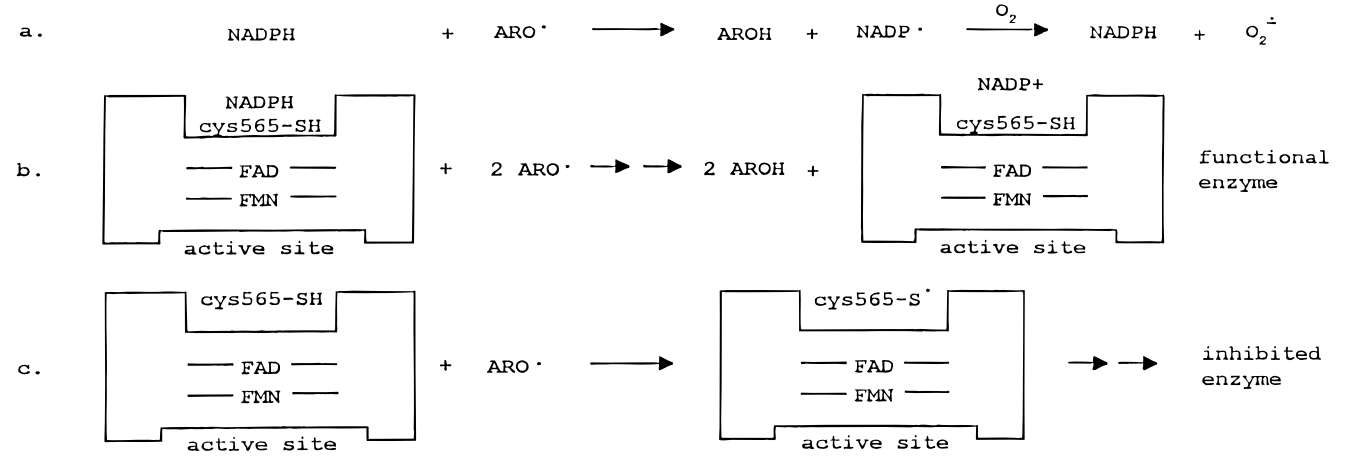
Scheme 1




in liver microsomes (Packer et al., 1989). In contrast, the rate of reduction of phenoxyl radical of phenol by NAD(P)H is $1 \times 10^8$ $M^{-1}$ $s^{-1}$ (Grodkowski et al., 1983) which is fast even without enzymatic catalysis.

In addition to NAD(P)H, phenoxyl radicals derived from various aryl alcohols can oxidize GSH (Wardman & von Sonntag, 1995; Sipe et al., 1994) and protein-SH (Goldman et al., 1995; Tyurina et al., 1995). Human OR is sensitive to inhibition by thiol reagents due to modification of cysteine 565 in the NADPH binding domain (Haniu et al., 1989). The OR could therefore be inhibited by phenoxyl radicals reacting with cysteine 565.

It is not clear if the OR will transfer electrons to phenoxyl radicals, but its ability to transfer reducing equivalents to substrates in one electron steps was clearly demonstrated (Vermilion et al., 1981). It is also not clear if the transfer would proceed catalytically from NADPH via FAD/FMN of the reductase or from cysteines of the flavoprotein with subsequent loss of activity. The possible reactions are summarized in Scheme 1.

This study examines the reactions of phenoxyl radicals of three phenolic compounds with OR and NADPH. The three phenols, in the order of increasing reduction potential (Wardman, 1989), were the following: (1) PMC (water soluble analog of α-tocopherol), $E^{\circ\prime} = 0.48$ V; (2) etoposide, $E^{\circ\prime} = 0.56$ V; and (3) phenol, $E^{\circ\prime} = 0.9$ V. A higher reduction potential indicates a stronger oxidant and correlates with a faster rate of reaction with reductants (Lind et al., 1990). In particular, this study examined the following: (1) if the decay of the relatively stable phenoxyl radical of PMC could be catalyzed by OR; (2) if the phenoxyl radical of VP-16 is more reactive and oxidizes NADPH directly; and (3) if the phenoxyl radicals of PMC, VP-16, or phenol could attack cysteine residues of the enzyme and inactivate the OR.

## EXPERIMENTAL PROCEDURES

### *Materials*

Recombinant human cytochrome P-450 reductase was purified from Sf9 cells heterologously transfected with baculovirus expression vector (see Methods), etoposide was a generous gift from Bristol-Myers Squibb (Syracuse, NY), methanol, ethanol, and acetic acid were from Fisher Scientific (Pittsburgh, PA), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, VA), and the protein assay kit and Chelex-100 resin were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Water was distilled in quartz glassware. Aqueous buffers and solutions were treated with Chelex-100 prior to all experiments.

### *Methods*

*Construction of Recombinant Baculovirus.* Recombinant baculovirus was constructed according to the previously described protocol (Gonzalez et al., 1991). Briefly, the OR cDNA was removed from pUC9 vector using *Eco*R1, and the Klenow fragment of DNA polymerase was used to make the fragment blunt ended. The insert was ligated to the transfer plasmid pAc373 that had been digested with *Bam*HI and made blunt ended. The pAc373 plasmid bearing the OR cDNA was co-transfected with baculovirus DNA onto *Spodoptera frugiperda* (Sf9) cells. The recombinant viruses were selected and purified by four rounds of plaque purification procedure. A $^{32}$P-nick-translated OR cDNA was used as the probe. The virus titer was determined as described previously (Gonzalez et al., 1991). To increase the level of expression, Sf9 cells were infected with the recombinant virus in spinner flasks at a multiplicity of infection of three plaque-forming units/cell.

*Human Cytochrome P-450 Oxidoreductase (OR).* The human OR was purified to apparent homogeneity from Sf9 cells infected for 72 h with recombinant baculovirus expression vector. Cells were washed three times with isotonic saline in phosphate buffer, pH 7.2, lysed by homogenization, and centrifuged at 1500*g* for 15 min at 4 °C. The supernatant was further centrifuged at 100000*g* for 70 min at 4 °C, the "microsomal" pellet was resuspended in 10 mM Tris-HCl, pH 7.5, containing 20% glycerol and 0.1 mM EDTA. Protein solubilization was achieved by addition of Emulgen 913 (Kao-Atlas, Tokyo) to final concentration of 1%. The solution was centrifuged for 60 min at 4 °C, and the supernatant was used to purify the OR using DEAE-Sepharose and 2′,5′-ADP-Sepharose chromatography as described previously (Yasukochi and Masters, 1976) with the following modification. The SDS−PAGE/Western Blot revealed two polypeptides of ∼70 and ∼8 kDa (data not shown). In order to remove a smaller polypeptide of hydrophobic tail, octyl-Sepharose was finally used. Spectral analysis of the purified OR showed a mixture of semiquinone

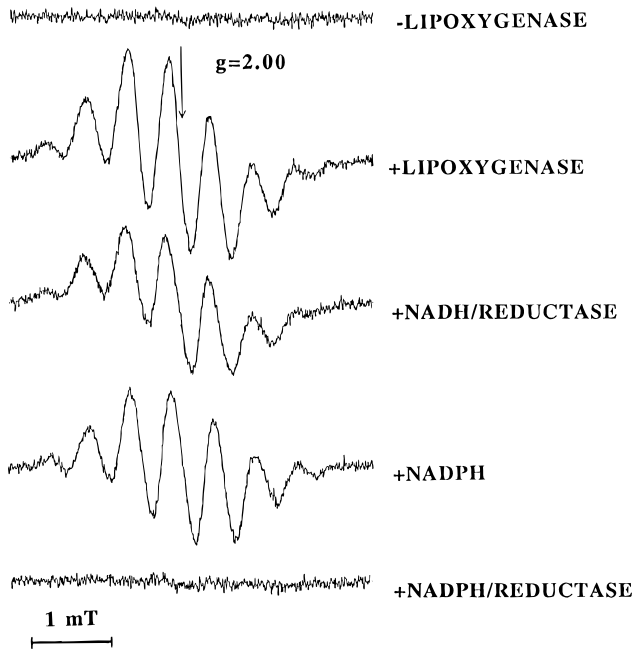



FIGURE 1: Quenching of the PMC-phenoxyl radical EPR signal by NAD(P)H and human NADPH-cytochrome P-450 reductase. PMC (10 mM) was oxidized to its phenoxyl radical in DOPC liposomes (20 mg/mL) by soybean lipoxygenase (30 units/$\mu$L) and arachidonic acid (0.05 mM). NADH (0.3 mM), NADPH (0.3 mM), and OR (0.1 units/mL) were added as indicated in the figure. EPR spectra were recorded as described in Methods. Presented is a typical of two experiments.

and oxidized forms which converted to fully oxidized form by addition of ferricyanide. Visible spectrum of this OR was typical of the flavoprotein purified from mammalian liver (Yasukochi & Masters, 1976) and the peak ratio $A_{275}/A_{455}$ was 7.48 (data not shown). Thus, we obtained the truncated flavoprotein which did not support monooxygenase reactions catalyzed by cytochrome P-450, although the OR was capable of reducing cytochrome *c*, DCPIP, and other soluble one-electron acceptors. It is noteworthy that a truncated form of OR with similar characteristics was isolated from the cytosol of HL-60 cells (Nisimoto et al., 1993). Activity of the reductase was determined spectrophotometrically by reduction of 0.05 mM DCPIP ($\lambda_{max} = 605$ nm, $\epsilon = 21\,000\ M^{-1}\ cm^{-1}$) in the presence of 0.5 mM NADPH (Inano & Tamoki, 1986). The content of the OR was 14.3 nmol/mg [using $\epsilon = 23.5\ mM^{-1}\ cm^{-1}$ at 455 nm and $M(r)$ 70 kDa], and its specific activity was 12.4 $\mu$mol/min/mg of protein at 25 °C in 0.1 M phosphate buffer, pH 7.4.

*(1) Reaction with PMC.* PMC (10 mM) was oxidized to its phenoxyl radical in DOPC liposomes (20 mg/mL) in phosphate buffer, pH 7.4, at 25 °C by soybean lipoxygenase (30 units/$\mu$L) and arachidonic acid (0.05 mM). NADH (0.3 mM), NADPH (0.3 mM), and OR (0.1 units/mL) were added as indicated in Figure 1, and EPR spectra were recorded immediately after the respective additions (see below).

*(2) Reaction with AAPH.* AAPH, 100 mM, and OR, 11.4 $\mu$g (~0.15 nmol), were incubated at 37 °C in 0.2 mL of phosphate buffer, pH 7.4. The time course of OR inactivation was determined by measurement of activity at given time points in 25 $\mu$L aliquots of the reaction diluted as needed. Protection of the enzyme was achieved by addition of etoposide (0.3 mM).

*(3) Reaction with Phenol/HRP/$H_2O_2$.* HRP type VI-A (0.3 $\mu$g/mL), $H_2O_2$ (0.2 mM), and OR (9.1 $\mu$g, ~0.12 nmol) were incubated at 27 °C in 0.1 M phosphate buffer, pH 7.4, with or without phenol (0.15 mM). Aliquots were withdrawn at given time points, reaction was stopped by catalase (300 units/mL), and activity was determined as described above. Protection of the enzyme by $NADP^+$ was examined by addition of $NADP^+$ (0.5 mM) to OR one minute prior to other reagents. In some experiments, DTT (10 mM) was added after termination of the reactions to determine if activity could be recovered. The activity of reductase was measured after 10 min of incubation with DTT at 27 °C as described above.

*(4) Reaction with Etoposide/HRP/$H_2O_2$.* Etoposide (0.3 mM) was oxidized to phenoxyl radicals by HRP (0.25−2.5 $\mu$g/mL) and $H_2O_2$ (0.2 mM). Reaction with OR and its activity were measured as described above for phenol.

*(5) Reaction with DTNB.* OR, 11.4 $\mu$g (~0.15 nmol), was first incubated with DTNB (0.4 mM) for 5 min. The mixture was split into halves and either distilled $H_2O$ ($dH_2O$) or DTT (10 mM) was added for another 5 min at 25 °C. Modified OR was separated from low $M(r)$ compounds on a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer, pH 7.4, and aliquots of the OR containing fraction were assayed for activity as above.

In another experiment, inhibition of the OR by DTNB was used to protect the enzyme from inactivation by phenoxyl radicals. OR (35 $\mu$g) was first incubated with DTNB (0.4 mM) for 5 min. DTNB-inhibited OR was exposed to phenol (0.12 mM), HRP (0.15 $\mu$g/mL), and $H_2O_2$ (0.15 mM) at 27 °C. Aliquots were withdrawn, the reaction was stopped by catalase (300 units/mL), and DTT (10 mM) was added for 10 min. Modified OR was separated from low $M(r)$ compounds on a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer, pH 7.0, and aliquots of the OR containing fraction were assayed for activity as described above.

*(6) Reaction with Phenol Oxidation Products.* Phenol (0.15 mM), HRP (0.3 $\mu$g/mL), and $H_2O_2$ (0.2 mM) were incubated at 27 °C in 0.1 M phosphate buffer, pH 7.4, for 7 and 15 min. Reaction was stopped with catalase (300 units/mL), and reductase (9.1 $\mu$g, ~0.12 nmol) was added to the mixture. Aliquots were withdrawn at given time intervals, and activity was determined as described above.

*Detection of Radical Intermediates by EPR Spectroscopy.* EPR measurements were performed on a JEOL-RE1X spectrometer at 20−37 °C in gas permeable Teflon tubing (0.8 mm internal diameter, 1.3 $\mu$m thickness, Alpha Wire Co., Elizabeth, NJ). The tubing was filled with 60 $\mu$L of aqueous sample, folded into quarters, and placed in an open EPR quartz tube in such a way that all the sample was within the effective microwave irradiation area. Spectra were recorded at 3355 G center field, 20−100 mW power, 0.5−2.0 G modulation, and 10−50 G/min scan time. Spectra were either plotted on a chart recorder or collected using EPRWare software (Scientific Software Services, Bloomington, IL) and transferred as ASCII files to EPR analysis software provided by the Free Radical Metabolite Research Group (NIEHS, Research Triangle Park, NC) for spectral analysis.

*HPLC Detection of Etoposide.* Aliquots of reaction mixtures withdrawn at times indicated in the figures were mixed with an equal volume of $CH_3OH$, passed through a C-18 cartridge (1mL Sep-Pac cartridge, Waters Division of Millipore Co., Milford, MA), and injected into a 20 $\mu$L loop

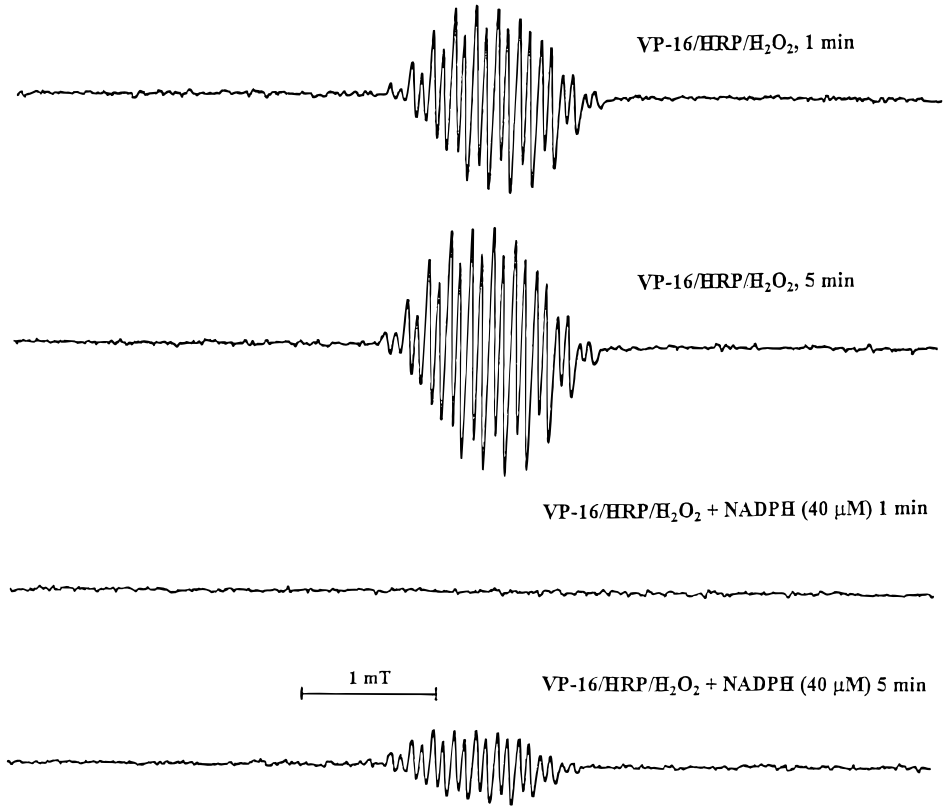



FIGURE 2: Quenching of the etoposide-phenoxyl radical EPR signal by NAD(P)H. VP-16 (0.15 mM) was oxidized by HRP (0.9 $\mu$g/mL) and $H_2O_2$ (0.25 mM) in 0.1 M phosphate buffer, pH 7.4, at 27 °C. NADPH (0.04 mM) was added as indicated and EPR spectra were recorded as described in Methods. Data presented are typical of three experiments.

of a rheodyne injector of a LC-10A HPLC with LC-600 pump and M10A photodiode array detector (Shimadzu, Kyoto, Japan). Etoposide was detected by UV absorption at 284 nm. The separation of etoposide from other reagents was achieved on a C-18 reverse phase column (Ultrasphere ODS, 5 $\mu$m particle size, 4.6 × 250 mm, Beckman) using a mobile phase of $CH_3OH$:$H_2O$ (11:9) adjusted to pH 3.1 with $CH_3CO_2H$ at a flow rate of 1 mL/min. Under these conditions the retention time of etoposide was 6 min.

*UV-Vis Spectrophotometric Measurements.* All spectrophotometric measurements were carried out using an UV160U spectrophotometer (Shimadzu, Kyoto, Japan) interfaced to a 486/33 Dell personal computer. The concentration of NADPH was measured at 340 nm, $\epsilon = 6200\ M^{-1}\ cm^{-1}$. The concentration of thiols was determined using Ellman's procedure (Ellman, 1959) by measuring absorption of reduced dithionitrobenzoic acid (DTNB) at 412 nm, $\epsilon = 13\,600\ M^{-1}\ cm^{-1}$. Phenol was monitored at 270 nm, $\epsilon = 14\,500\ M^{-1}\ cm^{-1}$, and phenol oxidation products were monitored at 400 nm.

## RESULTS AND DISCUSSION

Various enzymes (peroxidases, tyrosinase, lipoxygenase) catalyze oxidation of phenolic compounds to their respective phenoxyl radicals (O'Brien, 1988; Kagan et al., 1994). Phenoxyl radicals were detected in cultured mammalian cells exposed to phenolic xenobiotics (Schreiber et al., 1989). Since phenoxyl radicals can oxidize cysteine residues of GSH and proteins (Wardman & von Sonntag, 1995), their formation in cells could result in inactivation of enzymes sensitive to redox modifications. The rate of thiol oxidation by phenoxyl radicals is, in general, faster for phenoxyl radicals with higher reduction potential (Surdhar & Armstrong, 1986). The rates of reduction by thiols become insignificant for some stabilized phenoxyl radicals, e.g., the phenoxyl radical of α-tocopherol (Davies et al., 1988).

OR is sensitive to inactivation by SH-modifying reagents (Haniu et al., 1989). This flavoprotein has also the ability to catalyze NADPH-dependent reduction of various electron acceptors (see above). In this study, the ability of the OR to catalyze transfer of electrons from NADPH to phenoxyl radicals was therefore compared with inhibition of OR by phenoxyl radicals capable of modifying its cysteine residues.

*(1) Quenching of EPR Signal of PMC by OR/NAD(P)H.* PMC incorporated into DOPC liposomes was oxidized to its phenoxyl radical by soybean lipoxygenase/arachidonic acid as described previously (Packer et al., 1989). Steady state concentration of the phenoxyl radical was measured by the intensity of its EPR signal. In the absence of reductants, the signal reached steady state within 1 min and remained unchanged for at least 30 min.

Addition of NADH or NADPH decreased only slightly the intensity of the steady state EPR signal of the phenoxyl radical of PMC (Figure 1). The slow rate of reaction of the phenoxyl radical of PMC with NAD(P)H (Davies et al., 1988) was reflected in the inability of NAD(P)H to quench directly EPR signal of the phenoxyl radical of PMC (Figure 1).

When NADPH was added together with OR, the EPR signal of the phenoxyl radical of PMC was immediately quenched (Figure 1). The signal of the PMC phenoxyl radical reappeared in the spectrum upon prolonged incubation (data not shown) suggesting that lipoxygenase was not

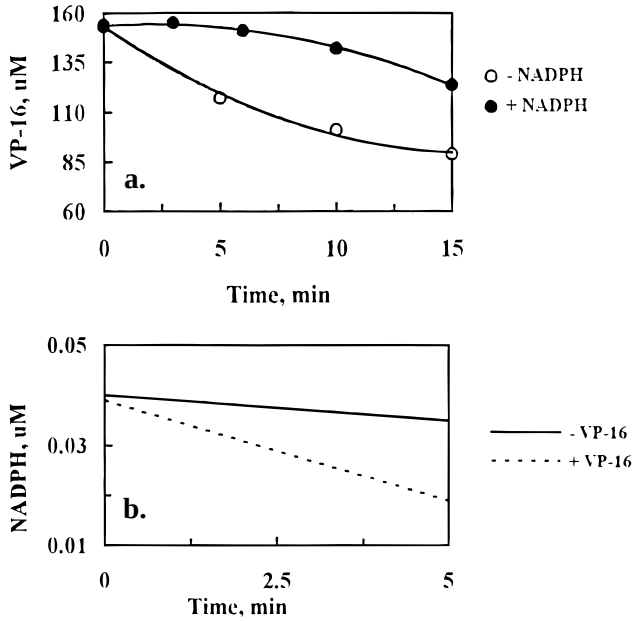



FIGURE 3: (a) Time course of oxidation of VP-16. VP-16 (0.15 mM) was oxidized by HRP (0.5 $\mu$g/mL) and $H_2O_2$ (0.25 mM) in 0.1 M phosphate buffer, pH 7.4, with NADPH (0.04 mM) (full circles) or without NADPH (open circles). Concentrations were determined by HPLC as described in Methods. Data presented are typical of three experiments. (b) Time course of oxidation of NADPH. NADPH (0.04 mM) was oxidized by HRP (0.5 $\mu$g/mL) and $H_2O_2$ (0.25 mM) in 0.1 M phosphate buffer, pH 7.4, with VP-16 (0.15 mM) (interrupted line) or without VP-16 (continuous line). Concentration was determined by continuous monitoring of absorbency at 340 nm. Data presented are typical of three experiments.

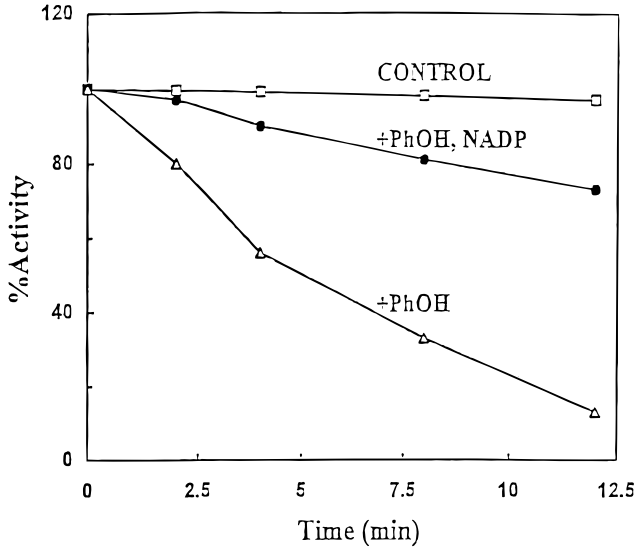



FIGURE 4: Inhibition of human NADPH-cytochrome P-450 reductase by phenol/$H_2O_2$/HRP. OR (9.1 $\mu$g), peroxidase (0.3 $\mu$g/mL), phenol (0.15 mM), and $H_2O_2$ (0.2 mM) were reacted at 27 °C in 0.1 M phosphate buffer, pH 7.4. Aliquots of the reaction were taken at times indicated in the figure, reaction was stopped by addition of catalase (300 units/mL), and activity was determined as described in Methods. Protection of OR from inhibition was analyzed under identical conditions with $NADP^+$ (0.5 mM) added to the OR 1 min prior to other reagents. Data presented are typical of four experiments.

inhibited. NADH did not support this reaction, which showed clearly that catalytic activity of the OR is involved (Scheme 1b). In liver microsomes, the quenching of the EPR signal of PMC phenoxyl radical is catalyzed by enzymes (Packer et al., 1989). Our EPR measurements suggest that OR could contribute to this activity (Figure 4).

*(2) Reaction of Etoposide Phenoxyl Radical with NADPH.* Etoposide was oxidized to its phenoxyl radical by HRP/$H_2O_2$ as described previously (Kalyanaraman et al., 1989). The EPR signal of the phenoxyl radical in the absence of NADPH reached steady state within 3 min and remained unchanged for at least 15 min (Figure 2). Addition of NADPH to the reaction at 5 min of incubation resulted in immediate quenching of the etoposide radical signal (Figure 2). The disappearance of the signal was transient (Figure 2) and the signal reappeared after an interval of 5 min (11 min total incubation time). The duration of this interval was proportional to the concentration of NADPH (data not shown). The reappearance of the radical signal (Figure 2) indicated that HRP was not inhibited by NADPH. The OR was not needed for the reduction of phenoxyl radical of etoposide by NADPH likely due to direct quenching by NADPH.

This assumption was further strengthened by the concurrent measurements of NADPH and etoposide oxidation. Measurements of NADPH concentration showed that the rate of its oxidation by HRP/$H_2O_2$ was slow (Figure 3b). The rate increased approximately 3-fold with the addition of etoposide. At the same time, addition of NADPH considerably delayed oxidation of etoposide by HRP/$H_2O_2$ (Figure 3a). This suggests that NADPH did not compete with etoposide for HRP but reduced the phenoxyl radical formed by HRP/$H_2O_2$.

*(3) Reaction of OR with the Phenoxyl Radical of PMC, Etoposide, and Phenol.* Activity of the OR was not modified by addition of any of the phenols studied without activation of the phenols by HRP/$H_2O_2$ or AAPH. Addition of either etoposide or PMC to HRP/$H_2O_2$ and OR for 15 min did not decrease activity of the OR (data not shown). Activity of the OR remained also unchanged during 15 min of incubation with HRP/$H_2O_2$ at 25 °C (Figure 4).

The activity of the OR decreased, however, in a time dependent fashion when phenol was added to HRP/$H_2O_2$ (Figure 4). This was expected on the basis of the demonstrated ability of phenoxyl radicals of phenol to oxidize protein thiols (Goldman et al., 1995). The decrease in activity was not reversible by removal of the reagents from the OR on a Sephadex G-25 column; the activity was also not recovered by addition of DTT to the inhibited OR (data not shown). Inhibition of the OR was prevented almost completely by addition of $NADP^+$ prior to the activation of phenol with HRP/$H_2O_2$ (Figure 4). Since $NADP^+$ (contrary to NADPH) does not reduce phenoxyl radicals of phenol, the protection was likely mediated by its binding to cytochrome P-450 reductase. This is in agreement with previous results which showed that cys565 of the human OR is located in the NADPH binding region and that the inactivation of the OR mediated by modification of cyc565 can be prevented by saturation of the enzyme with $NADP^+$ (Haniu et al., 1989).

It has been shown previously that low $M(r)$ thiols (Surdhar & Armstrong, 1986) including GSH (Wardman & von Sonntag, 1995) and protein-SH (Goldman et al., 1995) reduce phenoxyl radical of phenol in a one-electron reaction forming thiyl radicals. Oxidation of cys565 by phenoxyl radicals of phenol could be therefore responsible for the observed inhibition of the human OR (Scheme 1c). The oxidation of the disulfide forming protein, thioredoxin, is fully reversible

Table 1: Inhibition of NADPH-Cytochrome P-450 Reductase by AAPH[a]

| | reductase activity (nmol/min) | | | |
|---|---|---|---|---|
| time (min) | control | AAPH | AAPH+PhOH | AAPH+VP-16 |
| 0 | 33 ± 4 | 31 ± 5 | 35 ± 7 | 34 ± 5 |
| 15 | 31 ± 8 | 13 ± 7[b] | 15 ± 8[b] | 29 ± 6 |
| 30 | 27 ± 7 | 3 ± 5[b] | 6 ± 5[b] | 25 ± 8 |

[a] OR (11.4 $\mu$g) was reacted with AAPH (100 mM), phenol (0.3 mM), and VP-16 (0.3 mM) at 37 °C in 0.1 M phosphate buffer. Control, OR without AAPH at 37 °C. Aliquots were taken at times indicated in the table and analyzed for activity as described in Methods. Results represent average ± standard deviation of three measurements. [b] Significantly different from time zero at the 95% confidence level using two-way analysis of variance.

with DTT (Goldman et al., 1995). Attempts to reverse the inhibition of OR with DTT were not successful; hence formation of intramolecular or intermolecular disulfide involving cys565 is not likely to mediate the inhibition of the OR. Therefore, we examined whether the OR was inhibited by the products of phenol oxidation (see Methods). In all measurements, the OR retained more than 95% of its activity under these conditions (data not shown). The experiments supported the hypothesis that the phenoxyl radicals of phenol were responsible for the observed inhibition. We did not examine what was the final modification of OR responsible for the loss of activity. It is possible that cys565 was oxidized to thiyl radical (Goldman et al., 1995) with subsequent oxygenation (Zhang et al., 1994) or intramolecular transfer of the radical (Prutz et al., 1989).

*(4) Effect of VP-16 and Phenol on Inhibition of OR by AAPH.* The OR was inhibited in a time-dependent fashion by radicals derived from the thermal decomposition of AAPH (Niki, 1986) (Table 1). The OR was almost completely inhibited during 30 min of incubation with 100 mM AAPH at 37 °C. Phenols are efficient scavengers of peroxyl radicals (Foti et al., 1994). It has been shown previously that carbon-centered radicals, as well as alkoxyl and peroxyl radicals derived from AAPH (Krainev et al., 1996, Misik et al., 1996), are efficiently scavenged by phenolic compounds (Ritov et al., 1995). During the reaction, phenoxyl radicals of the respective phenols are formed. In agreement with the results on activation of phenols by HRP/$H_2O_2$ (Figure 4), etoposide fully protected the OR from inactivation by AAPH while phenol did not (Table 1). The result further supports the assumption that oxidation of phenol to phenoxyl radicals is responsible for the inhibition of the OR (Scheme 1c).

*(5) Inhibition of the OR by DTNB and Protection of the Inhibited OR from Inactivation by Phenol/HRP/$H_2O_2$.* Addition of DTNB resulted in loss of activity of the OR (Table 2) likely due to formation of mixed disulfide involving cys565 (Haniu et al., 1989). Removal of DTNB on a Sephadex G-25 column did not restore activity of the OR, but the activity could be recovered by the addition of DTT to the DTNB-inactivated OR. DTT alone did not modify activity of the OR (data not shown). When the DTNB inactivated OR was exposed to phenol/HRP/$H_2O_2$, its activity could be restored by subsequent addition of DTT (Table 2). Addition of DTT to OR inhibited by phenol/HRP/$H_2O_2$ (reductase was not treated with DTNB) did not restore activity of the enzyme (see above). The data indicate that phenol/$H_2O_2$/HRP (likely the phenoxyl radical of phenol) reacted with the same residue of the OR as DTNB (likely cys565 of the enzyme). The DTNB-inhibited OR was simply deprotected (reduced) by addition of DTT to remove DTNB from cys565.

Table 2: Protection of NADPH-Cytochrome P-450 Reductase from Inhibition by Phenol/HRP/$H_2O_2$ with DTNB[a]

| | OR activity (% CONTROL) | | | | |
|---|---|---|---|---|---|
| time (min) | DTT | DTNB | DTNB+ DTT | HRP+ DTT | HRP-DTNB+ DTT |
| 0 | 100 | 100 | 100 | 100 | 100 |
| 5 | 98 | ND | ND | 77 | 101 |
| 10 | 103 | 8 | 96 | 67 | 98 |

[a] OR (11.4 $\mu$g) was reacted with DTT (10 mM) (column 2) or DTNB (0.5 mM) (column 3) at 25 °C in 0.1 M phosphate buffer, pH 7.2, for 5 min. DTNB-inhibited OR was reacted for an additional 5 min with DTT (10 mM) (column 4). An aliquot of OR (34 $\mu$g) was first inhibited by DTNB (as in column 2). OR-DTNB (column 6) and an equal amount of unmodified OR (column 5) were reacted with peroxidase (0.15 $\mu$g/mL), phenol (0.12 mM), and $H_2O_2$ (0.15 mM) for 5 and 10 min, and the reaction was stopped with catalase (300 units/mL). DTT (10 mM) was added for another 10 min. Activity was measured after separation of OR from low *M*(*r*) reductants on an S-G-25 column. The result is typical of two experiments.

In summary, the results show that OR facilitates quenching of the EPR signal of PMC by NADPH. The phenoxyl radicals of etoposide and phenol are better oxidants than the radical of PMC and oxidize NADPH directly in the absence of the OR. In the absence of NADPH, OR is inhibited during activation of phenol by HRP/$H_2O_2$ or by AAPH. Modification of cys565 of the OR, involved in the binding of NADPH, mediates likely inhibition of the OR by phenol/$H_2O_2$/peroxidase because addition of $NADP^+$ (whose binding site contains cys565) and preincubation with DTNB (which forms probably a mixed disulfide with cys565) protects the enzyme from inactivation. The inhibition is likely due to phenoxyl radicals since the enzyme is not inhibited by products of phenol oxidation.

The inhibition of OR by phenoxyl radicals is selective. Irrespective of the source of oxidation, activation of etoposide and PMC does not inhibit the OR. Selective inactivation by phenol shows that (1) the site is not easily accessible (PMC and VP-16 radicals cannot reach cys565) and (2) oxidation of cys565 requires phenoxyl radicals with higher redox potential then etoposide.

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