Biochemical and Biophysical Research Communications **270,** 1086–1092 (2000)
doi:10.1006/bbrc.2000.2564, available online at http://www.idealibrary.com on IDEAL®

Myeloperoxidase-Catalyzed Phenoxyl Radicals of Vitamin E Homologue, 2,2,5,7,8-Pentamethyl-6-hydroxychromane, Do Not Induce Oxidative Stress in Live HL-60 Cells

Valerian E. Kagan,*,† Alexander I. Kuzmenko,*,†,‡ Anna A. Shvedova,§ Elena R. Kisin,§ Yulia Y. Tyurina,*,[1] and Jack C. Yalowich†

**Department of Environmental and Occupational Health and †Department of Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania 15238; §Health Effects Laboratory Division, Pathology and Physiology Research Branch, NIOSH, Morgantown, West Virginia 26505; and ‡A. V. Palladin Institute of Biochemistry, Ukrainian National Academy of Sciences, Kiev, 252030, Ukraine*

Received March 24, 2000

We used myeloperoxidase-containing HL-60 cells to generate phenoxyl radicals from nontoxic concentrations of a vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) to test whether these radicals can induce oxidative stress in a physiological intracellular environment. In the presence of H_2O_2, we were able to generate steady-state concentrations of PMC phenoxyl radicals readily detectable by EPR in viable HL-60 cells. In HL-60 cells pretreated with succinylacetone, an inhibitor of heme synthesis, a greater than 4-fold decrease in myeloperoxidase activity resulted in a dramatically decreased steady-state concentrations of PMC phenoxyl radicals hardly detectable in EPR spectra. We further conducted sensitive measurements of GSH oxidation and protein sulfhydryl oxidation as well as peroxidation in different classes of membrane phospholipids in HL-60 cells. We found that conditions compatible with the generation and detection of PMC phenoxyl radicals were not associated with either oxidation of GSH, protein SH-groups or phospholipid peroxidation. We conclude that PMC phenoxyl radicals do not induce oxidative stress under physiological conditions in contrast to their ability to cause lipid peroxidation in isolated lipoproteins *in vitro.*

Key Words: **myeloperoxidase; phenoxyl radicals; vitamin E homologues; HL-60 cells; GSH; protein SH groups; phospholipid peroxidation; oxidative stress.**

Vitamin E is the major lipid-soluble antioxidant of lipoproteins and biomembranes (1). As a phenolic compound (Ph-OH) its antioxidant activity is due to the ability to donate hydrogen from the hydroxy group of the chromanol moiety to reactive lipid chain-propagating radicals such as peroxyl radicals ($LOO^{\bullet}$) to yield lipid hydroperoxide (LOOH) and vitamin E phenoxyl radical ($Ph\text{-}O^{\bullet}$) (2):

$$LOO^{\bullet} + Ph\text{-}OH \xrightarrow{k_1} LOOH + Ph\text{-}O^{\bullet} \qquad [1]$$

The antioxidant effectiveness of vitamin E, however, depends not only on its reactivity toward lipid radicals (k_1) but also on the reactivity of its radical toward critical biomolecules in membranes and lipoproteins (3):

$$Ph\text{-}O^{\bullet} + LH \xrightarrow{k_2} Ph\text{-}OH + L^{\bullet}, \qquad [2]$$

where LH and $L^{\bullet}$ are lipid nonoxidized molecule and lipid alkyl radical, respectively.

Recently, Bowry and Stocker (4) reported that under some specific conditions (e.g., low rates of $LOO^{\bullet}$ generation) the reaction [2] may result in pro-oxidant rather than antioxidant effects of vitamin E in lipoproteins unless other reductants in the environment such as ascorbate (vitamin C) or ubiquinol (coenzyme Q) act as recyclers of vitamin E radical to prevent its pro-oxidant interactions with lipids. Moreover, (myelo)peroxidase-catalyzed generation of vitamin E phenoxyl radicals has been associated with induction of lipid peroxidation in lipoproteins (5, 6, 7). While these types of interactions have been unequivocally demonstrated in arti-

Abbreviations used: PMC, 2,2,5,7,8-pentamethyl-6-hydroxychromane; MPO, myeloperoxidase; GSH, glutathione; SA, 4,6-dioxoheptanoic acid (succinylacetone); PnA, *cis*-parinaric acid; 3-AT, 3-amino-1,2,4-triazole; PMSF, phenylmethylsulfonyl fluoride.

[1] On leave from Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, 194223, Russia.

0006-291X/00 $35.00

ficial model systems *in vitro*, their physiological or pathological significance, to the best of our knowledge, has not been demonstrated in either lipoproteins or in membranes.

With this in mind we designed experiments to determine whether enzymatic intracellular production of phenoxyl radicals will be accompanied by oxidative stress in intact cells. We incubated HL-60 cells (containing high endogenous myeloperoxidase (MPO) activity (8)) with nontoxic concentrations of a vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC), in the presence of hydrogen peroxide and conducted sensitive measurements of GSH and protein sulfhydryl oxidation as well as membrane peroxidation in different classes of phospholipids. We were able to directly detect, by EPR, MPO-catalyzed production of phenoxyl radicals that was not accompanied by any significant decrease of endogenous thiols or accumulation of phospholipid peroxidation in cells. We conclude that PMC phenoxyl radicals do not cause significant oxidative stress in HL-60 cells.

MATERIALS AND METHODS

Materials. 2,2,5,7,8-Pentamethyl-6-hydroxychromane was a gift from Eisai Co (Tokyo, Japan). *cis*-Parinaric acid (PnA) (Z-9, E-11, E-13, Z-15-octadecatetraenoic acid) was purchased from Molecular Probes, Inc. (Eugene, OR). The purity of each lot of PnA purchased was determined by UV spectrophotometry using the molar extinction ϵ_{304nm} in ethanol, 80×10^3 M^{-1} cm^{-1}. Hydrogen peroxide, 4,6-dioxoheptanoic acid (succinylacetone, SA), guaiacol, 3-amino-1,2,4-triazole (3-AT), phenylmethylsulfonyl fluoride (PMSF), glucose, cetylmethylammonium bromide, Hepes, fatty acid-free human serum albumin, malachite green base, sodium molybdate, sodium chloride, magnesium chloride, sodium phosphate, GSH, ethanol, lauryl sulfate, sodium salt (SDS), fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Iscove's medium was from GIBCO BRL (Grand Island, NY). Triton X-100 (*t*-octylphenoxypolyethoxyethanol) was from Bio-Rad Laboratories (Richmond, CA). 2-Propanol, methanol, hexane and acetonitrile were from Aldrich Chemical Co. (Milwaukee, WI). ThioGlo 1 was from Covalent Associates, Inc. (Woburn, MA). DMSO from Fisher Scientific Company (Pittsburgh, PA). All stock solutions were kept at −20°C. All other reagents were of the highest grade available.

Cell culture. Human promyelocytic HL-60 cells (from American Type Culture Collection) were grown in Iscove's medium supplemented with 15% FBS in 95% humidity atmosphere under 5% CO_2 in air at 37°C. Cells from passages 25–40 were used for the experiments. The density of cells at collection time was 0.5×10^6 cells/ml. PMC was dissolved in DMSO and added to cells (DMSO concentration not exceed 1%). SA was dissolved in Iscove's medium and added to cell culture (0.15×10^6 cells/ml) at the final concentration of 500 μM for 48 h. Cell viability was determined microscopically by trypan blue exclusion.

Myeloperoxidase activity. HL-60 cells were harvested by centrifugation at 1,000 rpm for 5 min. Pellets were washed twice with buffer A containing Hepes (25 mM), glucose (10 mM), NaCl (115 mM), KCl (5 mM), $MgCl_2$ (1 mM), NaH_2PO_4 (5 mM), pH 7.4. The homogenate was prepared by freezing at −77°C and thawing the cells. A spectrophotometric assay (Shimadzu UV 160U spectrophotometer (Kyoto, Japan)) of MPO activity was used in which guaiacol oxidation was monitored by changes of absorbance at 470 nm (ϵ = 26.6 mM^{-1} cm^{-1}) (9). Cell homogenate (0.5×10^6 cells) was added to Na,Na-phosphate buffer (100 mM) containing Triton X-100 (0.1%), PMSF (0.1 mM), guaiacol (13 mM), cetylmethylammonium bromide (0.02%), 3-AT (3.75 mM), pH 7.0. The reaction was started by the addition of H_2O_2 (670 μM). Activity of MPO was calculated in nmoles of tetraguaiacol per minute per 1×10^6 cells. The data were acquired using Shimadzu PC 160 software version 1.2.

EPR spectroscopy. Measurements of PMC phenoxyl radicals were performed on a JEOL-RE1X EPR spectrometer (Tokyo, Japan) at 25°C. Samples (50 μl) contained viable HL-60 cells (8×10^4 cells), PMC (100–400 μM), 3-AT (5 mM), H_2O_2 (5–600 μM). Measurements were done in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness) from Alpha Wire Corp. (Elizabeth, NJ). The tubing filled with mixed sample was folded twice and placed into a 3.0 mm EPR quartz tube. The EPR conditions were as follows: 335.7 mT center field, 10 mT sweep width, 0.2 mT field modulation, 10 mW microwave power, 0.3 s time constant, 4000 receiver gain, 1 min time scan. The kinetics of PMC phenoxyl radical formation was measured by repeated scanning of its EPR signal.

GSH measurement in HL-60 cells. GSH and protein SH groups in HL-60 cell homogenates were determined using a fluorogenic maleimide reagent, ThioGlo 1 as described earlier (10). ThioGlo 1 was dissolved in DMSO. The following conditions were used: homogenate (8×10^4 cells/ml), ThioGlo 1 (10 μM), Na,Na-phosphate buffer (100 mM), pH 7.4. Spectrofluorophotometer RF-5301 (Shimadzu, Kyoto, Japan) was employed for determination using an excitation slit of 1.5 nm and an emission slit of 5 nm. The wavelengths employed in the assay were 388 nm (excitation) and 500 nm (emission). The data were acquired using Shimadzu RF-5301PC software version 1.10.

HPLC assay of PMC. HPLC assay of PMC was performed as described earlier (11). Briefly, aliquots (200 μl) were taken at given time intervals and transferred into micro-centrifuge plastic tubes. For PMC extraction SDS (100 mM, 200 μl) was added to the samples, and briefly mixed by vortexing. Reagent alcohol (CH_3OH:C_2H_5OH, 1:1 by vol.) and hexane (400 μl each) were added and the resulting mixture was vigorously vortexed for 2 min. The mixture was centrifuged for 5 min at 1,000 rpm to separate the layers. The upper phase was transferred to a small tube and dried under N_2. The residue was redissolved in ethanol and analyzed by HPLC using the 5 μm, 4.6 × 200 mm ODS Hypersil (Hewlett Packard) column. A Waters HPLC system with 717 auto sampler, Waters 600 controller pump and a 474 Waters fluorescence detector were used. The wavelengths employed in the assay were 292 nm (excitation) and 324 (emission). The mobile phase used was CH_3CN/H_2O (8:5, by vol.) adjusted to pH 3.0 with CH_3COOH at a flow rate of 1 ml/min. Under these conditions, the retention time for PMC was 9.5 min. Control experiments showed that recovery of PMC with this procedure was >98% (11).

Determination of lipid peroxidation in HL-60 cells. PnA was incorporated into HL-60 cells by addition of complex PnA-human serum albumin to give a final concentration of 5 μg PnA/10^6 cells to serum free RPMI 1640 medium without phenol red as described earlier (12). PnA-labeled HL-60 were treated with H_2O_2 (200 μM) in the presence or in the absence of PMC (100 μM) for 15 min at 37°C in buffer A, pH 7.4 in the presence of 3-AT (5 mM). PMC was added to cell suspensions 10 min before addition of H_2O_2. At the end of incubation period, total lipids were extracted using Folch's procedure (13). The lipid extract was dried under N_2, dissolved in 0.2 ml of 2-propanol:hexane:water (4:3:0.16, by vol.) and separated by normal phase HPLC using a 5-μm Supelcosil LC-Si column (4.6 × 250 mm) and an ammonium acetate gradient as described early (12). The separations were performed using a Shimadzu HPLC system (LC-600) (Kyoto, Japan) equipped with an in-line configuration of RF-551 fluorescence detector. Fluorescence of PnA was measured at 420 nm emission after excitation at 324 nm. Data were processed and stored in digital form with Shimadzu EZChrom software. Lipid phosphorus was determined using a micro-method (14).

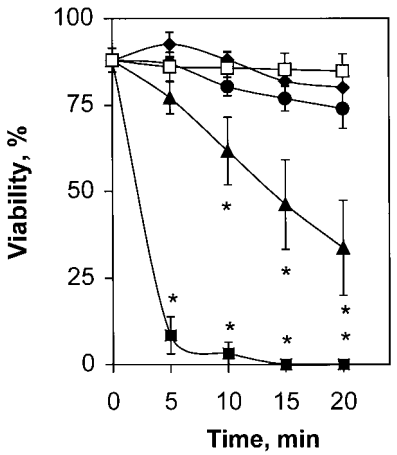

FIG. 1. Viability of HL-60 cells in the presence of different concentration of PMC. □, 200 μM H_2O_2 in the absence of PMC; ◆, 100 μM PMC + 200 μM H_2O_2; ●, 200 μM PMC + 200 μM H_2O_2; ▲, 300 μM PMC + 200 μM H_2O_2; ■, 400 μM PMC + 200 μM H_2O_2. HL-60 cells (1.6×10^6 cells/ml) were incubated with PMC (100–400 μM), 3-AT (5 mM) in buffer A (pH 7.4) in the absence or in the presence of H_2O_2 (200 μM) at 25°C. Viability was measured by trypan blue exclusion as described under Materials and Methods. Data are means ± SE, n = 4, *$p < 0.05$ *vs* HL-60 cells incubated in the absence of PMC.

Statistical analysis. The results are presented as mean ± SE values of at least three experiments and statistical analysis was performed by paired Student's *t*-test or one-way analysis of variance (ANOVA). The statistical significance of differences was set at $p < 0.05$.

RESULTS

Effect of PMC on HL-60 cells viability. PMC has been reported to induce Ca^{2+} permeability channels in membranes, hence causing cytotoxic effects (15). Since we were interested in the effects of PMC phenoxyl radicals in live cells, we tested the effects of various concentration of PMC on viability of HL-60 cells (Fig. 1). There were no statistically significant changes in viability of HL-60 cells exposed to 100 μM or 200 μM of PMC for 20 min either in the presence (Fig. 1) or in the absence (data not shown) of H_2O_2. At 300 μM, PMC caused a 2-fold decrease of HL-60 cell viability over 20 min incubation with 200 μM H_2O_2 (Fig. 1). A dramatic loss of viability was induced by 400 μM PMC independently of the presence (92% after 5 min of incubation) (Fig. 1) or absence (50% after 5 min of incubation) of H_2O_2 (data not shown). H_2O_2 alone (200 μM) did not cause any cytotoxic effects over 20 min incubation (Fig. 1).

PMC phenoxyl radicals are generated by MPO-catalyzed one-electron oxidation in viable HL-60 cells. Our previous work demonstrated that one-electron oxidation of PMC by tyrosinase (16), lipoxygenase (17) or horseradish peroxidase (18) yielded EPR-detectable phenoxyl radicals. In our initial experiments, we tested whether endogenous MPO in HL-60 cells can catalyze one-electron oxidation of PMC. A typical partially resolved EPR signal of PMC phenoxyl radicals (17) was detected upon addition of PMC to HL-60 cells in the presence of H_2O_2 and a catalase inhibitor, 3-AT (Fig. 2A). The magnitude of the EPR signal was dependent on PMC concentration (Fig. 2B). No EPR signals were observed when the cells were incubated with PMC in the absence of H_2O_2 or when PMC was incubated with H_2O_2 without cells (data not shown). Based on our results on cytotoxicity, 100 μM of PMC was chosen for further experiments despite the greater magnitude of the EPR signals at higher PMC concentrations.

As H_2O_2 is a co-substrate for MPO-catalyzed reactions, we tested whether the steady-state concentration of PMC phenoxyl radicals is dependent on H_2O_2 concentration. Using H_2O_2 at 200 μM, steady-state concentrations of PMC phenoxyl radicals were readily detectable by EPR and were maintained for ≈2 min and sharply decayed over subsequent 2 min time period (Fig. 3). The decay was due to H_2O_2 depletion as repeated addition of H_2O_2 reconstituted the magnitude of the signal (Fig. 3). Further increase of H_2O_2 concentration up to 400–600 μM only incrementally increased the duration of the PMC phenoxyl radicals (Fig. 3, inset). At very low H_2O_2 concentrations (5–10 μM), the EPR signal of the radical was detectable but rapidly decayed (within less than 1 min). The magnitude of PMC phenoxyl radical EPR signal was not substantially affected when an inhibitor of catalase, 3-AT was omitted from the incubation medium (data not shown), suggesting that PMC-driven consumption of H_2O_2 rather than its decomposition by catalase was the major pathway of its utilization in HL-60 cells

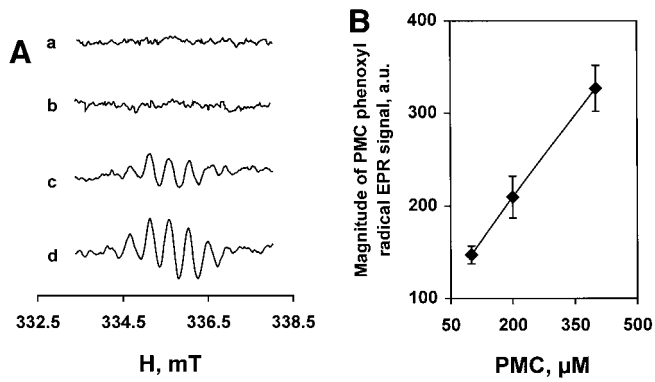

FIG. 2. MPO-catalyzed generation of PMC phenoxyl radicals in HL-60 cells. (A) EPR spectra of PMC phenoxyl radicals generated by MPO in HL-60 cells. a, cells + 3-AT (5 mM) + H_2O_2 (200 μM); b, cells + PMC (100 μM) + 3-AT (5 mM); c, cells + PMC (100 μM) + 3-AT (5 mM) + H_2O_2 (200 μM); d, cells + PMC (400 μM) + 3-AT (5 mM) + H_2O_2 (200 μM). (B) Dependence of PMC phenoxyl radical EPR signal on PMC concentration. HL-60 cells (1.6×10^6 cells/ml) were incubated in buffer A (pH 7.4) in the absence or in the presence of PMC and/or H_2O_2 at 25°C. EPR spectra of PMC phenoxyl radical were recorded as described under Materials and Methods. Data are means ± SE, n = 5.

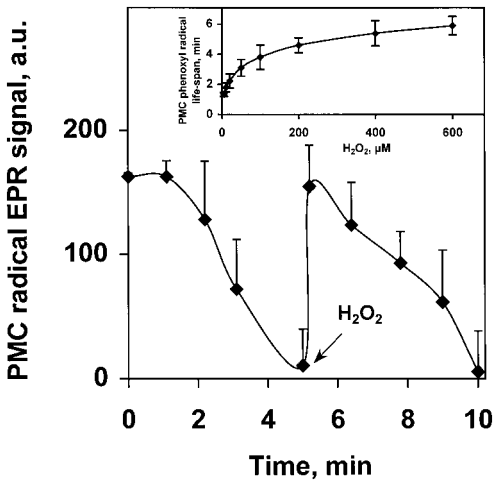

FIG. 3. Time course of PMC phenoxyl radical EPR signal. Arrow indicates second addition of H_2O_2. (Inset) The life span of PMC phenoxyl radical EPR signal at different concentrations of H_2O_2. HL-60 cells (1.6×10^6 cells/ml) were incubated with PMC (100 μM), 3-AT (5 mM) in buffer A (pH 7.4) in the presence of H_2O_2 (200 μM) at 25°C. EPR spectra of PMC phenoxyl radical were recorded as described under Materials and Methods. Time up to disappearance of EPR signal was calculated. Data are means ± SE, n = 5.

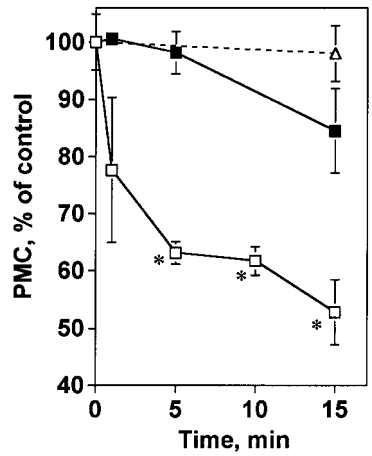

FIG. 5. H_2O_2-induced PMC oxidation in non-treated and SA-treated HL-60 cells. △, no H_2O_2; □, non-treated HL-60 cells; ■, SA-treated HL-60 cells (500 μM for 48 h). HL-60 cells (1.6×10^6 cells/ml) were incubated with PMC (100 μM), 3-AT (5 mM) in buffer A (pH 7.4) in the presence or in the absence of H_2O_2 at 25°C. H_2O_2 (200 μM) was added every 5 min. Aliquots of cells were taken at time points as indicated, and PMC contents was determined by HPLC as described under Materials and Methods. Data are means ± SE, *$p <$ 0.001 *vs* HL-60 cells incubated in the absence of H_2O_2.

under these conditions. Thus, 200 μM H_2O_2 was used in subsequent experiments.

To directly demonstrate the role of MPO activity in PMC radical formation in HL-60 cells we manipulated the enzymatic activity by growing the cells in the presence of an inhibitor of heme synthesis, SA. There were no significant changes in cell growth rate between control (non-treated) and SA-treated cells. After 48 h exposure to SA (500 μM), MPO activity in HL-60 cells significantly decreased (24.0 ± 2.2 *vs* 4.3 ± 0.1 nmoles guaiacol/min/10^6 cells, in control and treated cells, respectively, $p < 0.001$) (Fig. 4A). Under these conditions, PMC phenoxyl radical EPR signal was hardly detectable in the EPR spectra. Quantitatively, the magnitude of the signal was 19.5 ± 18.6 a.u. in SA-treated cells *vs* 170.7 ± 19.7 a.u. in non-treated cells (Fig. 4B).

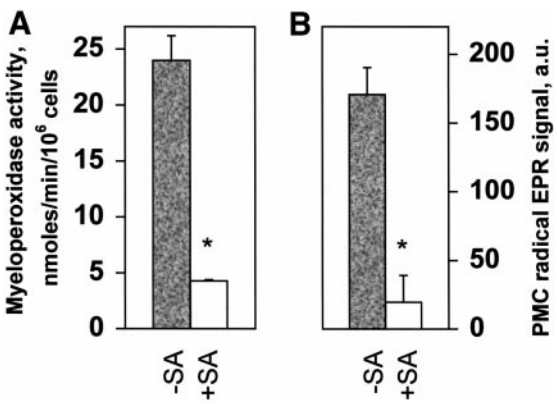

FIG. 4. Effect of SA on MPO activity (A) and EPR signal of PMC phenoxyl radicals (B) in HL-60 cells. MPO activity and EPR spectra of PMC phenoxyl radical were determined as described under Materials and Methods. Data are means ± SE, n = 3, *$p < 0.001$ *vs* HL-60 cells non-treated with SA.

HPLC measurements of PMC concentrations demonstrated that non-treated HL-60 cells consumed >45% of the compound over 15 min of incubation in the presence of H_2O_2 (Fig. 5). In contrast less than 15% PMC was oxidized when SA-treated HL-60 cells were incubated under the same conditions. Combined our results strongly suggest that MPO-catalyzed one-electron oxidation of PMC was responsible for the observed EPR signal of its phenoxyl radicals in HL-60 cells.

PMC phenoxyl radicals do not oxidize GSH and protein sulfhydryls in HL-60 cells. We next tested whether generation of PMC phenoxyl radicals was associated with oxidation of GSH or protein sulfhydryls. We found no decrease in GSH levels during 5 min incubation of HL-60 cells with PMC and H_2O_2 (Fig. 6) although PMC radical formation and PMC consumption was quite distinct during this period (see Figs. 2 and 5). Similarly, H_2O_2 alone did not cause changes in GSH content. A slight but insignificant decrease of GSH was detected in SA-treated HL-60 cells incubated with H_2O_2 alone which was not observed in the presence of PMC and H_2O_2 (Fig. 6A). These results indicate that MPO-catalyzed formation of PMC phenoxyl radicals was not associated with GSH oxidation.

Protein SH-groups were not substantially oxidized by H_2O_2 either in the presence or in the absence

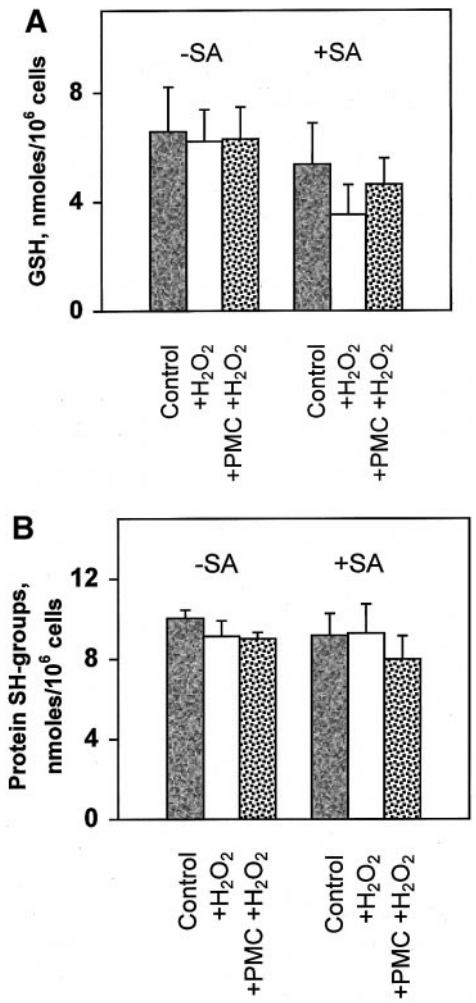

FIG. 6. Effect of PMC and H_2O_2 on the content of GSH (A) and protein sulfhydryls (B) in non-treated and SA-treated HL-60 cells. HL-60 cells (1.6×10^6 cells/ml) were incubated with 3-AT (5 mM) in buffer A (pH 7.4) in the presence or in the absence of PMC (100 μM) and/or H_2O_2 (200 μM) at 25°C. Aliquots of cells were taken at 5 min and GSH and protein sulfhydryls were determined by ThioGlo 1 as described under Materials and Methods. Data are means ± SE, n = 3.

of PMC in both non-treated and SA-treated cells (Fig. 6B).

PMC phenoxyl radicals do not induce lipid peroxidation in HL-60 cells. To determine whether production of PMC phenoxyl radicals induced lipid peroxidation in HL-60 cells we utilized our sensitive HPLC-fluorescent technique based on metabolic integration of oxidation-sensitive fluorescent fatty acid, *cis*-parinaric acid (PnA), in membrane phospholipids of HL-60 cells (12). We found that exposure HL-60 cells to H_2O_2 (200 μM) resulted in significant oxidation of phosphatidylinositol (PI) and phosphatidylserine (PS) in these cells (Fig. 7A). In SA-treated HL-60 cells, all four major classes of phospholipids were oxidized after H_2O_2 treatment (Fig. 7B). Essentially no significant peroxidation of phospholipids was detected after 15 min incubation of PnA-labeled cells when both H_2O_2 and PMC were present. Thus, PMC did not contribute to oxidative stress in PnA-labeled cells rather PMC protected phospholipids against oxidation induced by H_2O_2 alone in both non-treated and SA-treated HL-60 cells (Fig. 7). No peroxidation of phospholipids was induced when HL-60 cells were incubated with PMC alone (data not shown).

DISCUSSION

The present work demonstrates for the first time that endogenous MPO-catalyzed one-electron oxidation of a vitamin E homologue, PMC, to its phenoxyl radicals can be directly detectable by EPR in live HL-60 cells. This is strongly supported by our results demonstrating that SA—a potent inhibitor of 5-aminolevulinic acid dehydratase and hence of heme biosynthesis—resulted in a notable decrease of MPO activity and simultaneously in a dramatic quenching of the PMC phenoxyl radical EPR signal and inhibition of PMC oxidation as evidenced by our HPLC data. In line with this, we found that another phenolic compound, an antitumor drug etoposide (VP-16), was also converted by endogenous MPO into its phenoxyl radicals (Kagan *et al.*, in preparation). In this case, however, direct EPR detection of the radicals in HL-60 cells was only possible after depletion of endogenous GSH by reacting it with a maleimide reagent, ThioGlo-1 (19). This prompted us to further investigate possible interactions of PMC phenoxyl radicals with GSH and protein SH-groups in HL-60 cells.

We established that GSH was not consumed in either non-treated or SA-treated HL-60 cells in the presence of H_2O_2 alone or in combination with PMC. PMC also did not oxidize protein SH groups in the presence of H_2O_2 in either non-treated or SA-treated cells. Since the PMC phenoxyl radicals were directly observable in cells incubated with PMC and H_2O_2 we conclude that the radicals were not reduced and did not react with intracellular thiols. This is in sharp contrast to phenoxyl radicals of etoposide, that were highly reactive towards GSH resulting in a complete quenching of etoposide phenoxyl radical EPR signals by endogenous thiols (19).

Since recent reports implicated phenoxyl radicals of vitamin E and its homologues generated by (myelo)peroxidases in propagation of lipid peroxidation we decided to test whether peroxidation of membrane phospholipids was induced by endogenously formed PMC phenoxyl radicals. Our results unequivocally established that PMC phenoxyl radicals did not cause peroxidation in any of the four major classes of membrane phospholipids in HL-60 cells. Moreover, PMC protected against H_2O_2-induced peroxidation in both non-treated and SA-treated cells. This is specifically related to redox properties of phenoxyl radicals of vitamin E and its

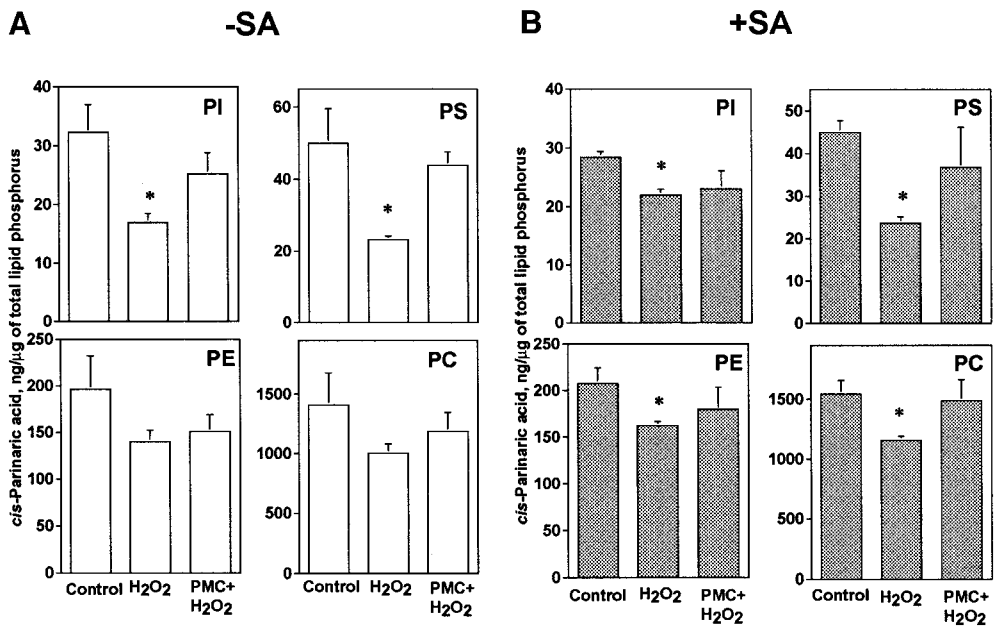

FIG. 7. Effect of PMC on oxidation of PnA-labeled phospholipid induced by H_2O_2 by HL-60 cells. (A) HL-60 cells; (B) HL-60 cells treated with SA for 48 h. *cis*-PnA-loaded cells (10^6 cells/ml) were exposure to H_2O_2 (200 μM) in the presence and absence of PMC (100 μM) in buffer A, pH 7.4 for 15 min at 37°C. PMC was added 10 min before addition of H_2O_2. At end time of incubation lipids were extracted and resolved by HPLC. Data are means ± SD, n = 3, *p < 0.03 *vs* control. *cis*-PnA, *cis*-parinaric acid; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

homologues rather than to particular localization of MPO in HL-60 cells as we found that MPO-catalyzed phenoxyl radicals of phenol induced extensive oxidation of membrane phospholipids in these cells (20).

Notably, no recycling of PMC from its phenoxyl radicals by endogenous reductants was observed in HL-60 cells under the conditions used as evidenced by our HPLC data. Since cell culture medium does not contain vitamin C recycling of PMC phenoxyl radicals by ascorbate (21) was not possible. Thus we conclude that even in the absence of ascorbate, prooxidant effects of PMC phenoxyl radicals towards either thiols or lipids are not realized in the normal intracellular environment of viable HL-60 cells.

Finally, the lack of prooxidant activity of PMC phenoxyl radicals opens avenues for the development of new chemopreventive strategies against cyto- and genotoxic effects of drugs whose calamitous mechanisms of action may be related to (myelo)peroxidase-catalyzed metabolism. In particular, we believe that our studies indicate new directions to overcome, at least to some degree, limitations in the clinical use of etoposide as a result of associated acute myelogenous leukemias (22, 23). This may be achieved by concomitant use of etoposide with vitamin E (its homologues) along with vitamin C and other vitamin E recycling agents to outcompete etoposide as a substrate for MPO and prevent the redox-cycling activity of its phenoxyl radicals in bone marrow progenitor cells.

ACKNOWLEDGMENTS

This work was supported by grants from the American Institute for Cancer Research 97-B128, NIH CA74972, CA77468, the Johns Hopkins Center for Alternatives to Animal Testing 9829 R3, and Leukemia Research Foundation and the International Neurological Science Fellowship Program (F05 NS 10669) administered by NIH/NINDS in collaboration with WHO, Unit of Neuroscience, Division of Mental Health and Prevention of Substance Abuse (Y.Y.T.).

REFERENCES

1. Packer, L., and Fuchs, J. (Eds.) (1993) Vitamin E in Health and Disease, pp. 1–1000. Dekker, New York/Basel/Hong Kong.
2. Burton, G. W., and Ingold, K. U. (1986) Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.* **19,** 194–201.
3. Mukai, K., and Okauchi, Y. (1989) Kinetic study of the reaction between tocopheroxyl radical and unsaturated fatty acid esters in benzene. *Lipids* **24,** 936–939.
4. Bowry, V. W., and Stocker, R. (1993) Tocopherol-mediated peroxidation. The pro-oxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *J. Am. Chem. Soc.* **115,** 6029–6044.
5. Santanam, N., and Parthaswarathy, S. (1995) Paradoxical actions of antioxidants in the oxidation of low density lipoprotein by peroxidases. *J. Clin. Invest.* **95,** 2594–2600.
6. Kalyanaraman, B., Darley-Usmar, V., Struck, A., Hogg, N., and Parthaswarathy, S. (1995) Role of apolipoprotein B-derived radical and α-tocopheroxyl radical in peroxidase-dependent oxidation of low density lipoprotein. *J. Lipid Res.* **36,** 1037–1045.
7. Witting, P. K., Upston, J. M., and Stocker, R. (1997) The role of α-tocopheroxyl in the initiation of lipid peroxidation in human

low density lipoprotein exposed to horse radish peroxidase. *Biochemistry* **36,** 1251–1258.

8. Traweek, S. T., Liu, J., Braziel, R. M., Johnson, R. M., and Byrnes, R. K. (1995) Detection of myeloperoxidase gene expression in minimally differentiated acute myelogenous leukemia (AML-M0) using in situ hybridization. *Diagn. Mol. Pathol.* **4,** 212–219.
9. Nonaka, T., Mio, M., Doi, M., and Tasaka, K. (1992) Histamine-induced differentiation of HL-60 cells. The role of cAMP and protein kinase A. *Biochem. Pharmacol.* **44,** 1115–1121.
10. Langmuir, M. E., Yang, J.-R., LeCompte, K. A., and Durand, R. E. (1996) New thiol active fluorophores for intracellular thiols and glutathione measurement. *In* Fluorescence Microscopy and Fluorescent Probes (Slavik, J., Ed.), pp. 229–234, Plenum Press, New York.
11. Lang, J. K., Cohil, L., and Packer, L. (1986) Simultaneous determination of tocopherols, ubiquinols and ubiquinones in blood, plasma, tissue homogenates and subcellular fractions. *Anal. Biochem.* **157,** 106–116.
12. Kagan, V. E., Ritov, V. B., Tyurina, Y. Y., and Tyurin, V. A. (1998) Sensitive and specific fluorescent probing of oxidative stress in different classes of membrane phospholipids in live cells using metabolically integrated *cis*-parinaric acid. *Methods Mol. Biol.* **108,** 71–87.
13. Folch, J., Lees, M., and Sloan-Stanley, G. H. (1957) A simply method for isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226,** 497–509.
14. Chalvardjian, A., and Rubincki, E. (1970) Determination of lipid phosphorus in the nanomolar range. *Anal. Biochem.* **36,** 225–226.
15. Kagan, V. E., Serbinova, E. A., Novikov, K. N., Ritov, V. B., Kozlov, Y. P., and Stoychev, T. S. (1986) Toxic and protective effects of antioxidants in biomembranes. *Arch. Toxicol. (Suppl.)* **9,** 302–305.
16. Tyurina, Y. Y., Tyurin, V. A., Yalowich, J. C., Quinn, P. J., Claycamp, H. G., Shor, N. F., Pitt, B. R., and Kagan, V. E. (1995) Phenoxyl radicals of etoposide (VP-16) can directly oxidize intracellulae thiols: Protective versus damaging effects of phenolic antioxidants. *Toxicol. Appl. Pharmacol.* **131,** 227–288.
17. Kagan, V. E., Serbinova, E. A., and Packer, L. (1990) Recycling and antioxidant activity of tocopherol homologues of different hydrocarbon chain length in liver microsomes. *Arch. Biochem. Biophys.* **282,** 221–225.
18. Tyurin, V. A., Carta, G., Tyurina, Y. Y., Banni, S., Day, B. W., Corongiu, F. P., and Kagan, V. E. (1997) Peroxidase-catalyzed oxidation of β-carotene in HL-60 cells and in model system: Involvement of phenoxyl radicals. *Lipids* **32,** 131–142.
19. Kagan, V. E., Yalowich, J. C., Borisenko, G. G., Tyurina, Y. Y., Tyurin, V. A., Thampatty, P., and Fabisiak, J. P. (1999) Mechanism-based chemopreventive strategies against etoposide-induced acute myeloid leukemia: Free radical/antioxidant approach. *Mol. Pharmacol.* **56,** 494–506.
20. Goldman, R., Claycamp, G. H., Sweetland, M. A., Sedlov, A. V., Tyurin, V. A., Kisin, E. A., Tyurina, Y. Y., Ritov, V. B., Wenger, S. L., Grant, S. G., and Kagan, V. E. (1999) Myeloperoxidase-catalyzed redox-cycling of phenol promotes lipid peroxidation and thiol oxidation in HL-60 cells. *Free Radical Biol. Med.* **27,** 1050–1063.
21. Kagan, V. E., Serbinova, E. A., and Packer, L. (1990) Generation and recycling of radicals from phenolic antioxidants. *Arch. Biochem. Biophys.* **280,** 33–39.
22. Pui, C. H., Ribeiro, R. C., Hancock, M. L., Rivera, G. K., Evans, W. E., Raimondi, S. C., Head, D. R., Behm, F. G., Mahmoud, M. H., and Sandlund, J. T. (1991) Acute myeloid leukemia in children treated with epipodophyllotoxins for acute lymphoblastic leukemia. *N. Engl. J. Med.* **325,** 1682–1687.
23. Felix, C. A. (1998) Secondary leukemias induced by topoisomerase-targeted drugs. *Biochim. Biophys. Acta* **1400,** 233–255.