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Direct evidence for recycling of myeloperoxidase-catalyzed phenoxyl radicals of a vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxy chromane, by ascorbate/dihydrolipoate in living HL-60 cells

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Abstract

Myeloperoxidase (MPO)-catalyzed one-electron oxidation of endogenous phenolic constituents (e.g., antioxidants, hydroxylated metabolites) and exogenous compounds (e.g., drugs, environmental chemicals) generates free radical intermediates: phenoxyl radicals. Reduction of these intermediates by endogenous reductants, i.e. recycling, may enhance their antioxidant potential and/or prevent their potential cytotoxic and genotoxic effects. The goal of this work was to determine whether generation and recycling of MPO-catalyzed phenoxyl radicals of a vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC), by physiologically relevant intracellular reductants such as ascorbate/lipoate could be demonstrated in intact MPO-rich human leukemia HL-60 cells. A model system was developed to show that MPO/H_2O_2-catalyzed PMC phenoxyl radicals (PMC•) could be recycled by ascorbate or ascorbate/dihydrolipoic acid (DHLA) to regenerate the parent compound. Absorbance measurements demonstrated that ascorbate prevents net oxidation of PMC by recycling the phenoxyl radical back to the parent compound. The presence of DHLA in the reaction mixture containing ascorbate extended the recycling reaction through regeneration of ascorbate. DHLA alone was unable to prevent PMC oxidation. These conclusions were confirmed by direct detection of PMC• and ascorbate radicals formed during the time course of the reactions by EPR spectroscopy. Based on results in the model system, PMC• and ascorbate radicals were identified by EPR spectroscopy in ascorbate-loaded HL-60 cells after addition of H_2O_2 and the inhibitor of catalase, 3-aminotriazole (3-AT). The time course of PMC• and ascorbate radicals was found to follow the same reaction sequence as during their recycling in the model system. Recycling of PMC by ascorbate was also confirmed by HPLC assays in HL-60 cells. Pre-loading of HL-60 cells with lipoic acid regenerated ascorbate and thus increased the efficiency of ascorbate in recycling PMC•. Lipoic acid had no effect on PMC oxidation in the absence of ascorbate. Thus PMC phenoxyl radical does not directly oxidize thiols but can be recycled by dihydrolipoate in the presence of ascorbate. The role of phenoxyl radical recycling in maintaining antioxidant defense and protecting against cytotoxic and genotoxic phenolics is discussed.

Keywords: Antioxidant recycling; Vitamin E homologue; Ascorbic acid; HL-60 cell; Phenoxyl radical

Abbreviations: PMC, 2,2,5,7,8-pentamethyl-6-hydroxychromane; PMC•, PMC phenoxyl radicals; MPO, myeloperoxidase; DHLA, dihydrolipoic acid; GSH, glutathione; HL-60, human leukemia-60 cells; DHA, dehydroascorbic acid; 3-AT, 3-amino-1,2,4-triazole; SDS, sodium dodecyl sulfate

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1. Introduction

Vitamin E (α-tocopherol) is the major lipid-soluble antioxidant of lipoproteins and biomembranes [1]. As a phenolic compound (Ph-OH), its antioxidant activity relies on the ability to donate hydrogen from the hydroxyl group attached to the chromanol ring to reactive chain-propagating radicals, to yield the phenoxyl radical of α-tocopherol [2].

$$LOO^{\bullet} + Ph - OH \longrightarrow LOOH + Ph - O^{\bullet} \quad (1)$$

The efficacy of vitamin E as an antioxidant not only depends on its reactivity toward damaging radicals [1], but also on the relatively stable nature of its radical due to delocalization of the unpaired electron about the chromanol ring. This prevents its reaction with subcellular constituents such as intracellular thiols (e.g., GSH), carotenoids, membrane phospholipids and DNA [3,4]. One electron oxidation of phenolic compounds to their respective phenoxyl radicals is known to be catalyzed by cytochrome P450-dependent monooxygenases, peroxidases and tyrosinase [5–9]. Since vitamin E cannot be synthesized by mammals and must be supplied in the diet, it is a potentially limiting resource. It has been inferred that the vitamin is conserved by its recycling, i.e. reduction of the tocopherol phenoxyl radical by other antioxidants to regenerate the parent compound [10]. Several recycling pathways have been delineated using the water-soluble vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) [10–12]. PMC phenoxyl radicals can be reduced nonenzymatically by ascorbate [10] or enzymatically in coenzyme-Q-mediated electron transport reactions [13]. Antioxidant recycling of vitamin E and its homologue has been demonstrated in a variety of cell-free systems such as chemical solvents [11], liposomes [12,14], lipoproteins [15,16] and isolated microsomal and mitochondrial membrane preparations [17].

Recycling of phenoxyl radicals has yet to be demonstrated in living cells. In earlier studies from our laboratory, phenoxyl radicals of PMC have been identified in homogenates prepared from human leukemia HL-60 cells [18]. These cells have a relatively high myeloperoxidase (MPO) activity capable (in the presence of H_2O_2) of catalyzing one-electron oxidation of PMC to its phenoxyl radical. This feature was considered suitable for investigating the antioxidant recycling of phenolic compounds in a physiological environment [18]. Furthermore, it was observed that in HL-60 cell homogenates, PMC (in the presence of H_2O_2) effectively competes against other phenolic compounds as a substrate for MPO to prevent formation of harmful phenoxyl radicals that may directly interact with intracellular thiols and/or cause oxidative DNA damage.

The present study was undertaken to determine whether recycling of PMC phenoxyl radicals by physiologically relevant intracellular reductants such as ascorbate/lipoate could be demonstrated in living HL-60 cells. A model system was developed to show that MPO/H_2O_2-dependent oxidation of PMC could be recycled by ascorbate or dihydrolipoic acid (DHLA) to recover the parent compound. PMC phenoxyl radicals were identified in suspensions of live HL-60 cells and these radicals were recycled by intracellular ascorbate/DHLA.

2. Materials and methods

2.1. Materials

PMC was a gift from Eisai Co. (Tokyo, Japan). Hydrogen peroxide, MPO, guaiacol, 3-amino-1,2,4-triazole (3-AT), glucose, sodium dodecyl sulfate (SDS), HEPES, sodium chloride, sodium phosphate, ethanol and 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 (*tert*-octylphenoxylpolyethanol) was from Bio-Rad Laboratories (Richmond, CA). Methanol, hexane and acetonitrile were from Aldrich Chemical Co. (Milwaukee, WI). DMSO was from Fisher Scientific Company (Pittsburgh, PA). All stock solutions were kept at −20 °C. All other reagents were of the highest grade available.

2.2. Cell culture conditions

Human promyelocytic HL-60 cells (from American Type Culture Collection) were grown in Iscove's medium supplemented with 15% FBS in a 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.

2.3. MPO activity

HL-60 cells were harvested by centrifugation at 1000 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) and 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 in absorbance at 470 nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). Cell homogenate ($0.5 \times 10^6$ cells) was added to Na-phosphate buffer (100 mM) containing Triton X-100 (0.1%), PMSF (0.1 mM), guaiacol (13 mM), cetyltrimethylammonium bromide (0.02%), 3-AT (3.75 mM), pH 7.0. 3-AT, a heme enzyme inhibitor, was added to prevent consumption of H_2O_2 by catalase. Under our experimental conditions, 3-AT (up to 5 mM) inhibited MPO activity by 20–30% in cells while completely inhibiting catalase activity. The reaction was started by the addition of H_2O_2 (670 μM). Activity of MPO was calculated in nanomoles of tetraguaiacol formed

per minute per 10^6 cells. The data were acquired using Shimadzu PC 160 software version 1.2.

2.4. Concentration of ascorbate and PMC oxidation product

Ascorbate and PMC/PMC oxidation products have characteristic absorption peaks in the UV and these were used to monitor concentrations as a function of time. Absorbance peaks for ascorbate, PMC and PMC oxidation products are centered at 265, 292 and 274 nm, respectively. Spectra were recorded at 10-min intervals using the Shimadzu 160 UV–visible spectrophotometer interfaced to a Dell 486P/33 personal computer. In order to distinguish between the two overlapping peaks of ascorbate and PMC oxidation products in reaction mixtures containing both reagents, experiments were performed in two parts. During the initial phase of oxidation, PMC concentration remained constant but ascorbate concentration decreased. To clearly observe this decrease, without interference from any eventual PMC oxidation products, the reference cuvette contained 100 μM PMC. When ascorbate was exhausted the reference cuvette was changed to buffer so that kinetics of PMC• EPR spectra (second phase) could be observed.

2.5. Identification of radical species by EPR spectroscopy

PMC phenoxyl radicals and ascorbate radicals were detected using a JEOL-RE1X EPR spectrometer (Tokyo, Japan) at 25 °C. Samples (50 μl) contained 8×10^4 viable HL-60 cells, 100 μM, 3-AT (5 mM), H_2O_2 (600 μM). Samples from model systems additionally contained 0.3 U/

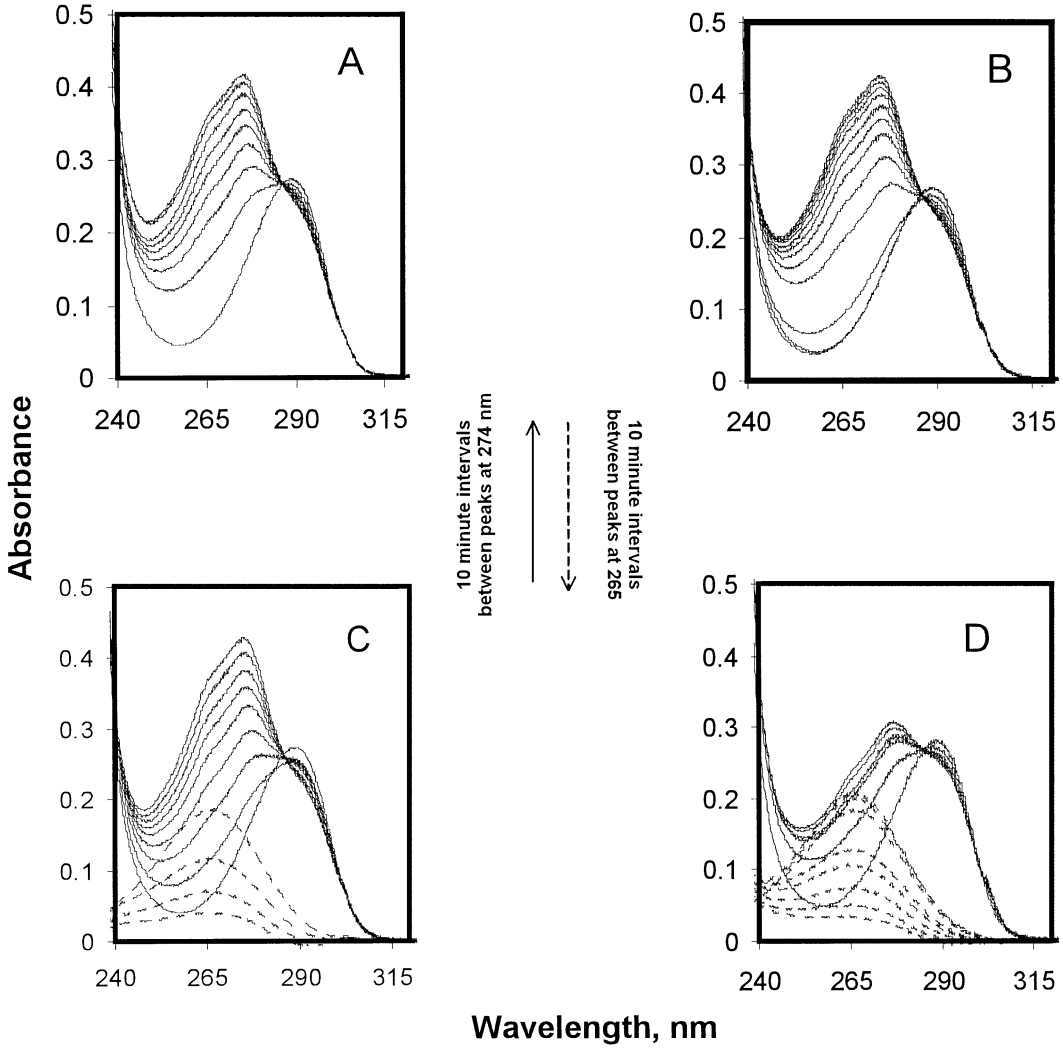


Fig. 1. Spectrophotometric assay of MPO/H_2O_2-induced PMC oxidation in the presence and absence of ascorbate and DHLA. (A) UV spectra of a reaction mixture consisting of PMC (100 μM) and 0.3 U/ml MPO in L1210 buffer, pH 7.4, were recorded at 10-min intervals after initiating the reaction by addition of 600 μM H_2O_2. (B) DHLA (4 μM) was added to the reaction mixture. (C) 16 μM ascorbate was added to the reaction mixture ± 100 μM PMC in the reference cuvette (see Section 2). (D) Ascorbate (16 μM) and DHLA (4 μM) were added to the reaction mixture under conditions described in (C). Spectra from PMC/PMC oxidation products (solid lines) show an increase in absorbance at 274 nm at each 10-min interval whereas spectra from ascorbate (dashed lines) show a decrease in absorbance at 265 nm at each 10-min interval.

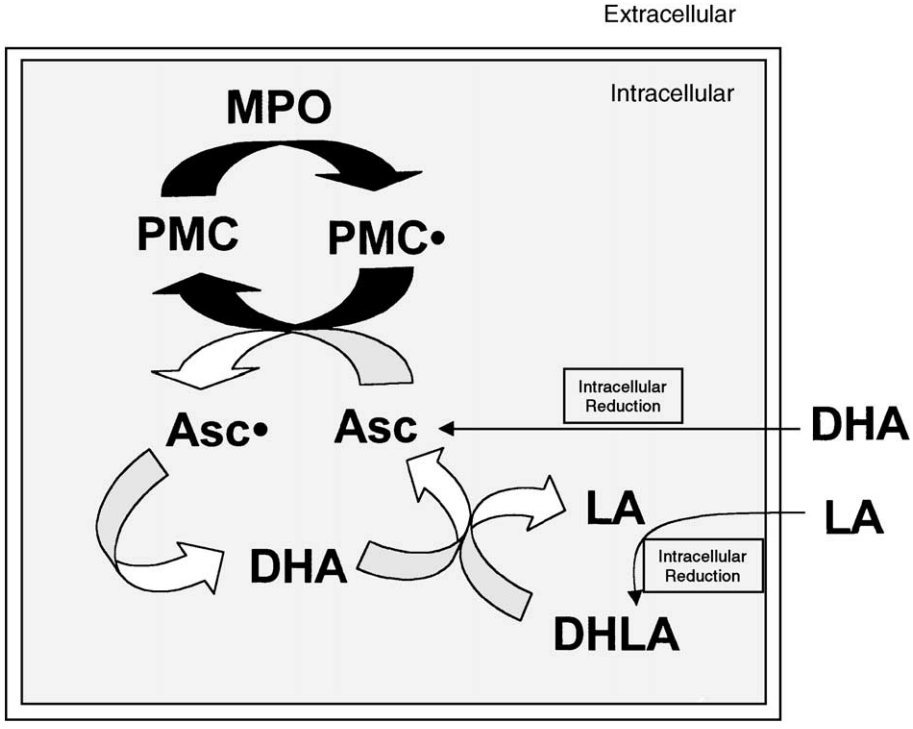
ml MPO but no 3-AT. For experiments involving ascorbate, HL-60 cells were pre-incubated for 2 h with dehydroascorbic acid (DHA) (31.2 μmol/10^6 cells), which is known to be reduced to ascorbate intracellularly [19]. Over this time, maximum DHA uptake facilitated by glucose transporters was estimated to yield intracellular ascorbate content of 3.1 ± 0.2 nmol/10^6 cells. Measurements were performed in gas-permeable Teflon tubing (0.8-mm internal diameter, 0.013-mm thickness) from Alpha Wire Corp. (Elizabeth, NJ). The tubing containing the sample was folded twice and placed into a 3.0-mm EPR quartz tube. The EPR conditions for detecting PMC phenoxyl radicals were as follows: 3357 G center field, 100 G sweep width, 2 G field modulation, 10 mW microwave power, 0.3 s time constant, 4000 receiver gain, 1 min time scan. The EPR condition for ascorbate radicals was: 3357 G center field, 25 G sweep width, 0.63 G field modulation, 10 mW microwave power, 0.1 s time constant, 4000 receiver gain, 1 min time scan.

2.6. HPLC assay of PMC

HPLC assay of PMC was performed as described earlier [17]. 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 mixed briefly by vortexing. Reagent Grade 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 1000 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 a 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 nm (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%.

2.7. HPLC determination of ascorbate

HPLC was performed essentially as described in Ref. [20]. After precipitation of proteins in homogenates by addition of 10% CCl_3CO_2H and sedimentation (2000 $g \times 10$ min), the supernatant was used for HPLC measurements. The column consisted of an 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-10 A HPLC system with UV detection (264 nm) was employed. The retention time for ascorbic acid was 3.4 min.



Scheme 1. MPO catalyzes the oxidation of PMC to a phenoxyl radical (PMC•). This radical is recycled to PMC by ascorbate, yielding ascorbate radical (Asc•) which disproportionates to yield ascorbate and dehydroascorbate (DHA). The latter is recycled to ascorbate by DHLA with concomitant production of lipoic acid (LA). Ascorbate and DHLA are delivered to cells in oxidized form to be reduced internally.

3. Results

3.1. Generation of PMC phenoxyl radicals by MPO in model system

To establish the parameters of an MPO-H_2O_2 oxidation system to generate PMC phenoxyl radicals, a model system was established [18]. The incubation of PMC in the presence of MPO-H_2O_2 resulted in rapid disappearance of PMC, as judged by absorbance at 292 nm, and the appearance of a new peak centered at 274 nm that characterizes oxidation products of PMC (Fig. 1). In the MPO-H_2O_2 system PMC is first oxidized to a short-lived PMC• (not detectable in UV spectra) then subsequently to orthoquinone [21,22] (Fig. 1A). An isobestic point is observed in the absorption spectra indicating a time-dependent conversion of PMC into a single oxidation product, i.e. PMC-orthoquinone [21,22]. The kinetics of the oxidation reaction was unaffected by the presence of DHLA (Fig. 1B) but the disappearance of PMC was prevented by reduced ascorbate (Fig. 1C). When all the ascorbate was oxidized (measured by disappearance of the absorbance peak at 265 nm), oxidation of PMC was again observed at a rate similar to that recorded in the absence of ascorbate. When DHLA was present in addition to ascorbate the rate of ascorbate oxidation slowed until its eventual exhaustion after about 1 h, after which PMC was oxidized (Fig. 1D). These results are consistent with a model in which phenoxyl radical intermediates of PMC oxidation by MPO-H_2O_2 are reduced by ascorbate to regenerate PMC. The reaction can be sustained in the presence of DHLA by reduction of ascorbate radicals produced when oxidation products of PMC are reduced during the recycling process (see Scheme 1).

The absorbance data plotted as a function of reaction time (Fig. 2) shows that no oxidation products of PMC appeared in reaction mixtures containing ascorbate until all the ascorbate has been oxidized. Since PMC oxidation was initiated in the absence of ascorbate (Fig. 2A) it may be concluded that ascorbate recycled PMC back to the parent compound. The action of DHLA on the time course of recycling is illustrated in Fig. 2B. The presence of DHLA essentially had no effect on the oxidation of PMC in the absence of ascorbate (compare open squares in Fig. 2A and B). While the rate of PMC oxidation in the presence of DHLA was slightly higher than in its absence, the difference did not reach the level of significance ($P < 0.1$ for the time points 10, 20, 30, 40 min of incubation). If DHLA were oxidized by PMC phenoxyl radicals it would be expected to diminish PMC oxidation. In the presence of ascorbate, DHLA slowed the net rate of ascorbate oxidation and appearance of oxidation products (compare closed circles Fig. 2A and B) by recycling oxidized ascorbate which in turn recycled oxidized PMC. We conducted measurements for PMC oxidation in the presence of DHLA ± ascorbate for additional 10 min (up to 90 min total). This was done to better illustrate PMC oxidation after a relatively long lag

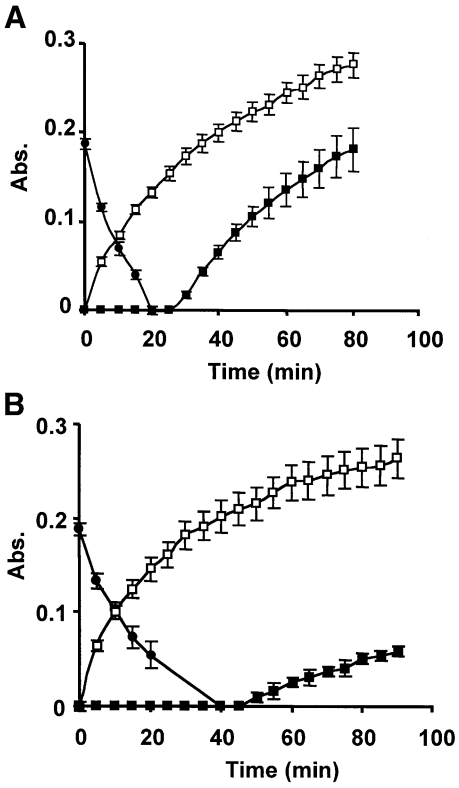


Fig. 2. Time course of MPO/H_2O_2-induced PMC oxidation in the presence and absence of ascorbate and DHLA. (A) Absorbance at 274 nm in reaction mixture without (open squares) and with (closed squares) 16 μM ascorbate. Absorbance at 265 nm due to ascorbate (closed circles). (B) Absorbance at 274 nm in reaction mixtures containing 4 μM DHLA without (open squares) and with (closed squares) 16 μM ascorbate. Absorbance at 265 nm due to ascorbate (closed circles).

period caused by DHLA-enhanced recycling of PMC in the presence of ascorbate. We attribute the slower initial rate of formation of PMC oxidation products in the presence of ascorbate + DHLA (Fig. 2B, closed squares) compared to ascorbate alone (Fig. 2A, closed squares) to a partial depletion of H_2O_2 during the longer period of time required to consume ascorbate (40–45 min in Fig. 2B compared to 20–25 min in Fig. 2A). The rate of PMC oxidation product formation was quite similar beyond 45 min whether or not ascorbate was added (compare open and closed squares in Fig. 2B), indicating that the addition of DHLA does not slow the rate of PMC oxidation. While reduction of PMC phenoxyl radicals by ascorbate/dihydrolipoate is a plausible explanation for these data, direct detection of the radical intermediates was required to provide additional support for this recycling system.

The identity of the radical species was confirmed by EPR spectroscopy and the results are summarized in Fig. 3. It can be seen that reaction of PMC in the presence of MPO and the co-substrate H_2O_2 produced a spectrum of a partially resolved phenoxyl radical, PMC$^{\cdot}$ (Fig. 3A). This signal was still present after 8 min and persisted in recordings made up to 14 min (data not shown). An almost identical pattern of

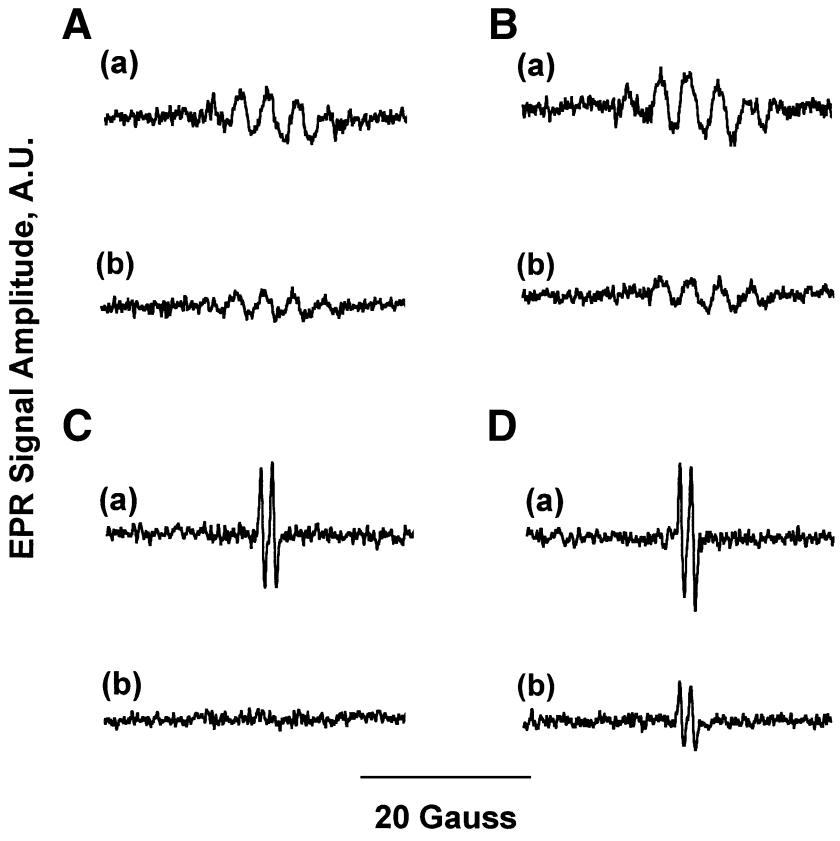


Fig. 3. EPR spectra of free radicals generated during MPO/H_2O_2-induced PMC oxidation in the presence and absence of ascorbate and DHLA. EPR spectra recorded 2 min (a) and 8 min (b) after initiation of the reaction (A). Spectra recorded from reaction mixtures containing 4 μM DHLA (B), 16 μM ascorbate (C) and 4 μM DHLA + 16 μM ascorbate (D).

spectral changes was observed in reaction mixtures containing 4 μM DHLA (Fig. 3B), indicating that DHLA did not reduce the one-electron PMC oxidation intermediate, PMC•. A strong radical signal, identified as ascorbate radical, was detected when the reaction mixture contained 16 μM ascorbate and there was no evidence of a signal from PMC• species (Fig. 3C). The signal from the ascorbate radical could not be detected 8 min after initiation of the reaction, and was replaced by the PMC• EPR signal. In reaction mixtures containing both DHLA and ascorbate, a strong ascorbate radical spectrum was recorded after 2 min which persisted beyond 8 min (Fig. 3D), suggesting that the presence of DHLA resulted in extended recycling of ascorbate. In the absence of MPO and adventitious metals the combination of ascorbate + H_2O_2 did not yield ascorbate radicals (not shown).

Changes in the time courses of radical formation (Fig. 4) follow a pattern similar to the changes in concentration of oxidation products of PMC and ascorbate measured by absorption spectroscopy (Fig. 2). A progressive decrease in the phenoxyl radical signal intensity from PMC• was seen during the first 14 min of the reaction with MPO–H_2O_2. (Fig. 4A) No phenoxyl radical was detected when an ascorbate radical EPR signal was present. Once this ascorbate radical EPR signal disappeared, the PMC phenoxyl radical EPR signal was detected at intensity similar to that recorded in the absence of ascorbate. Again the presence of DHLA in the reaction mixture has no effect on the intensity of the radical signal from PMC• unless ascorbate is also present. In this case the effect of DHLA was to prolong the existence of the ascorbate radical signal (Fig. 4B), resulting in a delay in the subsequent appearance of the PMC phenoxyl radical EPR signal (Fig. 4A).

We next investigated the possibility that ascorbate might compete with PMC as a substrate for MPO accounting for delayed appearance of the PMC radical EPR signal. For kinetic monitoring of the ascorbate and PMC radical formation by EPR spectroscopy, we repeatedly scanned a small part of the magnetic field (5 G) every 20 s over an 8-min time interval. Under these conditions, the entire doublet signal of the ascorbate radical EPR signal (aH = 1.79 G) was repeatedly observable in the time course recordings. However, for a broader and relatively poorly resolved PMC radical signal (≈ 30 G), only a portion of the entire signal could be accommodated within 5 G segment of the field. In a model system in the presence of ascorbate alone, MPO/H_2O_2 generated a very weak and long-lived (greater than 2 h) EPR signal of ascorbate radicals, consistent with ascorbate acting as a poor substrate for MPO under these conditions (Fig. 5A). When PMC was oxidized by the

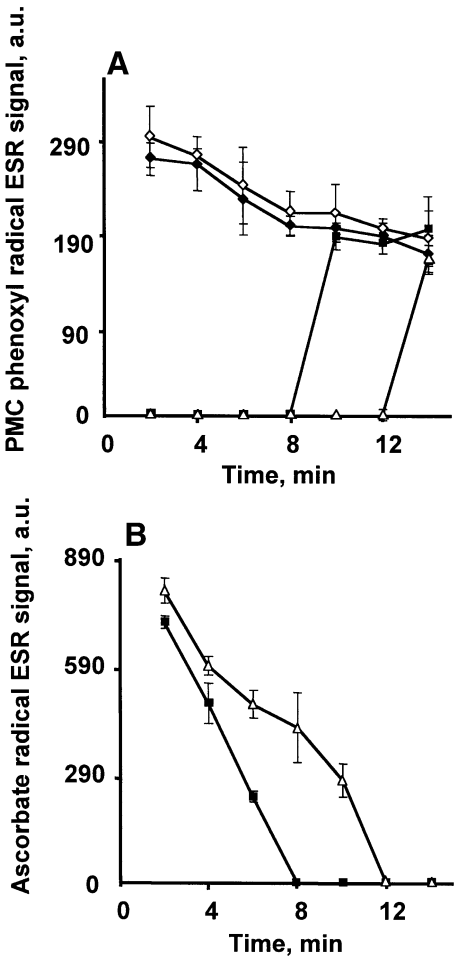


Fig. 4. Time course of EPR signals of ascorbate (B) and PMC (A) radicals in reaction mixtures containing MPO-H_2O_2 and either PMC with (open diamonds) or without (closed diamonds) 4 μM DHLA, or PMC + 16 μM ascorbic acid with (open triangles) or without (closed squares) 4 μM DHLA.

MPO/H_2O_2 system in the absence of ascorbate, a typical EPR signal of PMC phenoxyl radical was observed. MPO/H_2O_2-catalyzed oxidation of PMC in the presence of ascorbate yielded ascorbate radical EPR signals, which decayed, disappeared and were subsequently substituted by the PMC phenoxyl radical EPR signals. This suggests that ascorbate can effectively reduce PMC phenoxyl radicals, thus precluding the detection of PMC phenoxyl radicals during the time when ascorbate is still present. The magnitude of the ascorbate radical EPR signal was increased proportionally to the PMC concentration as would be expected as more ascorbate is utilized to recycle PMC phenoxyl radicals (Fig. 5B). If ascorbate were an effective substrate for MPO, then it would be expected that the ascorbate radical EPR signal would decrease, not increase, upon addition of a competitive substrate, PMC. In the presence of PMC, the ascorbate radical EPR signal decayed more rapidly as PMC concentrations were increased as reflected in the inverse relationship between PMC concentration and the life span of the ascorbate radical EPR signal (see Fig. 5B, inset). These results are consistent with a greater rate of depletion of ascorbate as PMC phenoxyl radicals are recycled.

3.2. Recycling of PMC radicals in human leukemic HL-60 cells in vivo

The above experiments demonstrate that MPO-H_2O_2 can oxidize PMC, the phenoxyl radical of which can be recycled by ascorbate back to the parent molecule in a model system.

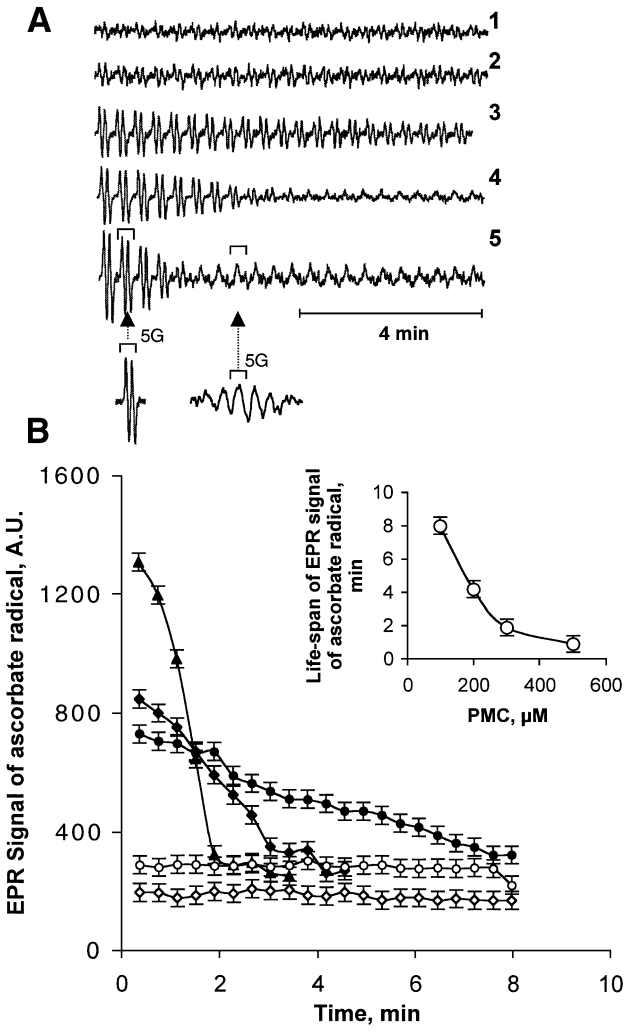


Fig. 5. Effect of PMC on the formation of ascorbate radicals in MPO/H_2O_2 model system. (A) Kinetic EPR recordings of the ascorbate radical and PMC radical EPR signals formed by MPO/H_2O_2. 1—ascorbate + MPO; 2—Ascorbate + MPO + H_2O_2; 3—ascorbate + MPO + H_2O_2 + PMC (100 μM); 4—ascorbate + MPO + H_2O_2 + PMC (200 μM); 5—ascorbate + MPO + H_2O_2 + PMC (300 μM). (B) Time course of ascorbate radical EPR signal in the presence and absence of PMC. Ascorbate + MPO (open diamonds); ascorbate + MPO + H_2O_2 (open circles); ascorbate + MPO + H_2O_2 + PMC (100 μM) (closed circles); ascorbate + MPO + H_2O_2 + PMC (200 μM) (closed diamonds); ascorbate + MPO + H_2O_2 + PMC (300 μM) (closed triangles). Inset: Effect of PMC on the life span of ascorbate radical EPR signal. Ascorbate (16 μM) and MPO (0.3 U/ml) were incubated in L1210 buffer, pH 7.4 containing 5 mM 3-AT, 600 μM H_2O_2 in the presence or absence of PMC (100–300 μM). Sixty-microliter samples were used for EPR measurements.

The next experiment was to determine whether the endogenous MPO in intact human leukemia HL-60 cells was able to generate radicals during oxidation of PMC in vivo and whether these radicals could be recycled by ascorbate. The endogenous MPO activity was measured in HL-60 cells in the absence and presence of recycling agents (lipoic acid or DHA) and the results are presented in Table 1. These results demonstrate that HL-60 cells have a relatively high MPO activity measured in the presence of co-substrate H_2O_2. Since the activity in cells (46.9 ± 2.5 nmol tetraguaiacol formed/min/ml) was comparable to that in the model system used (23.7 nmol tetraguaiacol formed/min/ml) the detection of PMC phenoxyl radicals and their recycling in cells seemed to be plausible. Preincubation with either lipoic acid or dehydroascorbate had no significant effect on the activity of MPO in HL-60 cells.

To determine whether endogenous MPO from intact cells was able to oxidize PMC upon addition of H_2O_2, EPR spectroscopy was used to detect PMC phenoxyl radical EPR signals. Results (Fig. 6) show that cells incubated with PMC and H_2O_2 in the presence of the inhibitor of catalase, 3-AT, gave rise to a signal of the partially resolved phenoxyl radical of PMC. This radical was observed 8 min after addition of H_2O_2 and was still detectable up to 14 min (Fig. 6A), after which no measurements were taken. No signals of PMC phenoxyl radicals were detected from HL-60 cells grown in the presence of an inhibitor of heme synthesis, succinyl acetone (results not shown), that resulted in a profound reduction of MPO activity in HL-60 cells (from 35.1 ± 1.9 to 4.3 ± 0.1 nmol tetraguaiacol formed/min/10^6 cells). These results indicate that MPO-catalyzed one-electron oxidation of PMC is indeed the source of PMC phenoxyl radicals.

A similar typical pattern of spectral features was recorded in cell suspensions preincubated with lipoic acid (Fig. 6B), suggesting that neither lipoic acid nor DHLA (formed as a result of its intracellular reduction) directly recycle PMC• produced by endogenous MPO from HL-60 cells. No phenoxyl radical spectra could be detected in HL-60 cell suspensions that had been preincubated with DHA. Instead a strong signal characterized as ascorbate radical was recorded (Fig. 6C) which progressively weakened with time and was not detectable after 10 min. At this time, a poorly resolved EPR signal of PMC• became detectable (data not shown). This suggests that uptake and intracellular reduction of dehydroascorbate to ascorbate provided for PMC• recycling in HL-60 cells. The ascorbate radical EPR signal remained strong if the cells were preincubated with lipoic acid in addition to dehydroascorbate (Fig. 6D). Lipoic acid has been shown to be reduced in cells to DHLA [23] indicating that the latter, as in the model system, regenerated ascorbate, providing a source of reducing equivalents to reduced PMC phenoxyl radicals back to PMC.

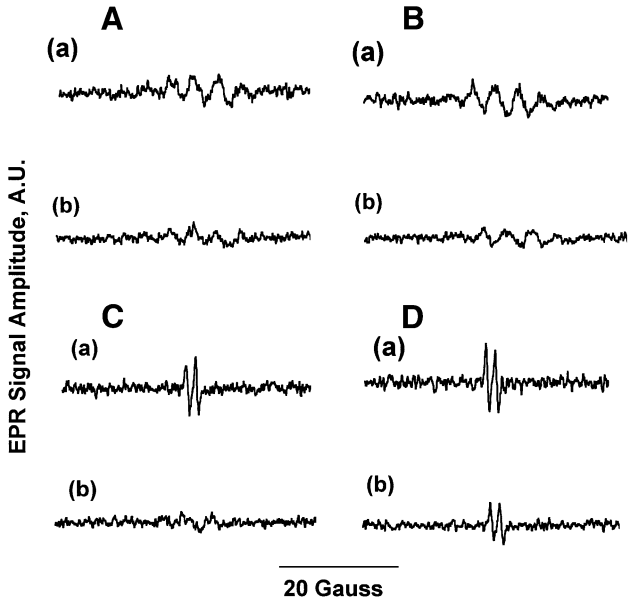


Fig. 6. Free radicals generated by a suspension of HL-60 cells incubated with 100 μM PMC, 600 μM H_2O_2 and 5 mM 3-AT. (A) EPR spectra recorded 2 min (a) and 8 min (b) after addition of H_2O_2. (B) EPR spectra recorded from cell suspensions as in (A) after loading with 10 μM lipoic acid. (C) EPR spectra recorded from cell suspensions as in (A) after preincubation with 50 mM dehydroascorbic acid for 1 h. (D) EPR spectra recorded from cell suspensions as in (A) after loading with 10 μM lipoic acid and 50 mM dehydroascorbic acid.

The time course of spectral intensity changes recorded for phenoxyl radicals of PMC and ascorbate radical in the HL-60 cell suspensions is presented in Fig. 7. The signal intensity from PMC phenoxyl radicals decreased slightly during the 14-min incubation following addition of this MPO co-substrate (Fig. 7B) but in cell suspensions that had been preincubated with DHA, no such signals could be detected (Fig. 7B) until the signal from ascorbate radical was lost (Fig. 7A). This is consistent with the pattern seen in the model system and indicates that ascorbate present inside the cells was responsible for recycling the phenoxyl radical of PMC to regenerate the parent compound. Lipoic acid had no effect on the generation of PMC$^{\cdot}$ catalyzed by endogenous MPO (Fig. 7B), but in the presence of intracellular ascorbic acid it was able to regenerate ascorbic acid, which in turn recycled PMC phenoxyl radicals (Fig. 7B).

In order to confirm that ascorbate was a poor MPO substrate and hence an unlikely competitor of PMC for MPO in intact HL-60 cells, cells were loaded with ascorbate after which ascorbate radical EPR signals were recorded in

Table 1
MPO activity of HL-60 cells

Treatment	MPO activity	
	nmol/min/10^6 cells	nmol/min/ml
Control	35.1 ± 1.9	46.9 ± 2.5
Succinyl acetone	4.3 ± 0.1	5.7 ± 0.1
Lipoic acid	33.3 ± 2.6	44.5 ± 3.5
Dehydroascorbic acid	34.5 ± 1.5	46.1 ± 2.0

Data are mean ± S.E., $n = 4$.

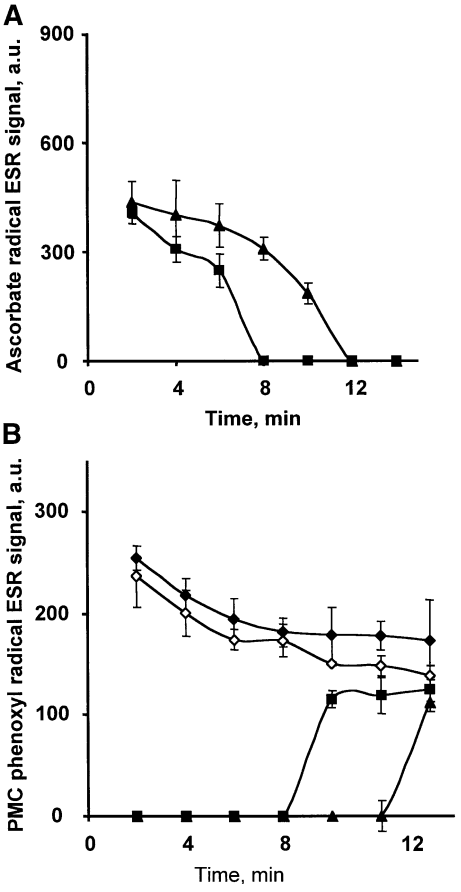


Fig. 7. Time course of free radicals generated by a suspension of HL-60 cells incubated with 100 μM PMC, 600 μM H_2O_2 and 5 mM 3-AT. Magnitudes of EPR signals of ascorbate radicals (A), with (closed triangles) or without (closed squares) 10 μM lipoic acid and from phenoxyl radicals of PMC (B), with (closed diamonds) or without (open diamonds) 10 μM lipoic acid, with dehydroascorbic acid (31.3 μmol/10^6 cells) (closed squares) or with a combination of lipoic acid and dehydroascorbic acid (at above concentrations) (closed triangles). Cell suspensions requiring dehydroascorbate and/or lipoic acid were preincubated as described in Section 2.

the absence or presence of various concentrations of PMC (Fig. 8). As in similar experiments using the MPO/H_2O_2 enzyme system (Fig. 5), there was a small long-lived ascorbate radical EPR signal in the presence of ascorbate alone (Fig. 8A) that increased proportionally to the concentration of PMC added (Fig. 8A and B). The decay rate and lifetime of the ascorbate radical EPR signals was inversely proportional to the PMC concentration (Fig. 8A, B and inset) consistent with ascorbate recycling of PMC phenoxyl radicals rather than ascorbate acting as a competitive substrate for MPO.

In addition to direct EPR detection of free radical intermediates, the recycling outcomes were investigated using HPLC for measurement of PMC and ascorbate in aliquots removed from cell suspensions during incubation in the presence of PMC and H_2O_2. Incubation of HL-60 cells with PMC in the presence of H_2O_2 caused a time-dependent consumption of the vitamin E homologue (Fig. 9). In succinyl acetone pretreated HL-60 cells (with lowered MPO activity, Table 1), a significantly lower rate of PMC consumption was observed (2.5- to 3-fold during 15 min of incubation, data not shown) indicating the predominant role of MPO in PMC oxidation. Dehydroascorbate prevented MPO-catalyzed loss of PMC during incubation over 10 min (Fig. 9). Since ascorbate was measured by HPLC, confirmation of the conversion of dehydroascorbate to ascorbate by the HL-60 cells was obtained. When lipoic acid was also present, the decrease in PMC concentration during the incubation was attenuated. These results are consistent with the previously mentioned recycling system demonstrated in HL-60 cells by EPR (see Scheme 1).

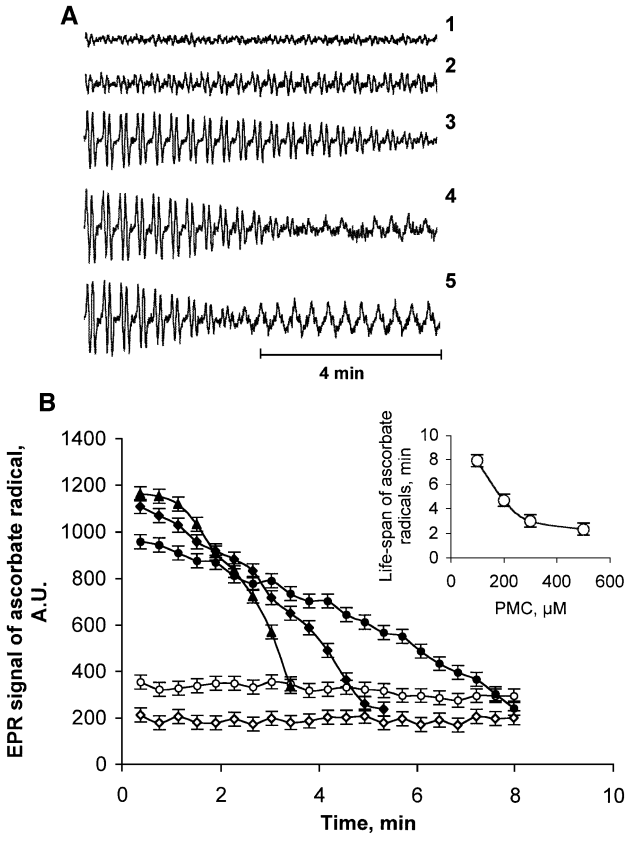


Fig. 8. Effect of PMC on the formation of ascorbate radicals in HL-60 cells. (A) Kinetic EPR recordings of the ascorbate radical and PMC radical EPR signals formed in HL-60 cells. 1—Control, HL-60 cells + 3-AT; 2—HL-60 cells + 3-AT + H_2O_2; 3—HL-60 cells + 3-AT + H_2O_2 + PMC (100 μM); 4—HL-60 cells + 3-AT + H_2O_2 + PMC (200 μM); 5—HL-60 cells + 3-AT + H_2O_2 + PMC (300 μM). (B) Time course of ascorbate radical EPR signal in HL-60 cells in the presence and absence of PMC. HL-60 cells + 3-AT (open diamonds); HL-60 cells + 3-AT + H_2O_2 (open circles); HL-60 cells + 3-AT + H_2O_2 + PMC (100 μM) (closed circles); HL-60 cells + 3-AT + H_2O_2 + PMC (200 μM) (closed diamonds); HL-60 cells + 3-AT + H_2O_2 + PMC (300 μM) (closed triangles). Inset: Effect of PMC on the life span of ascorbate radical EPR signal. HL-60 cells were preincubated for 2 h at 37 °C in the absence or presence of dehydroascorbic acid (31.2 μmol/10^6 cells) in L1210 buffer, pH 7.4. Cells were then washed and resuspended in PBS containing 5 mM 3-AT, 600 μM H_2O_2 in the presence or absence of PMC (100–300 μM). Sixty-microliter samples of HL-60 cells (8 x 10^4 cells) were used for EPR measurements.

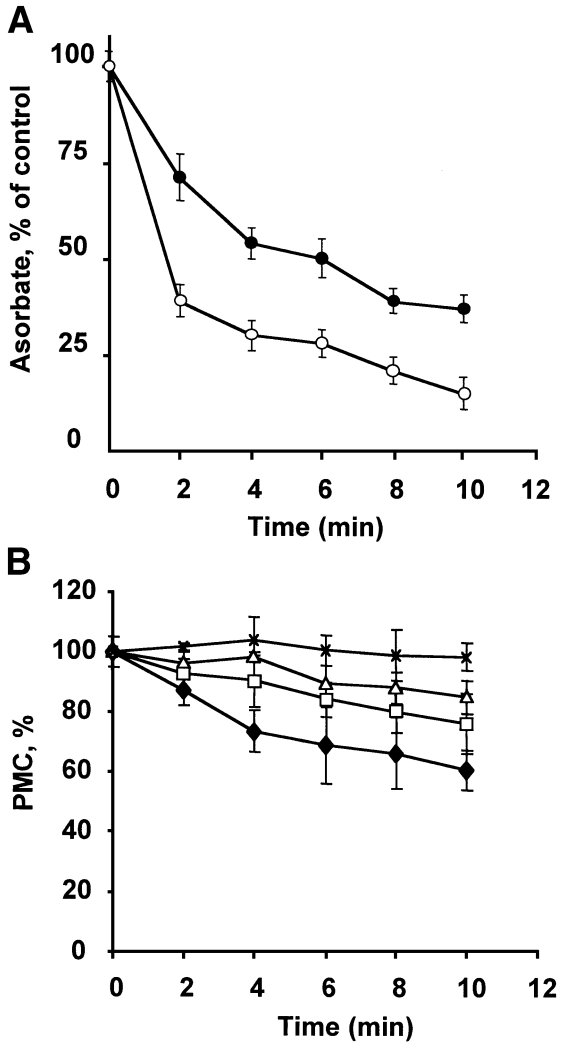


Fig. 9. Effect of lipoic acid on oxidation of PMC and ascorbate in HL-60 cells. HL-60 cells were pre-incubated for 2 h at 37 °C in the absence or presence of dehydroascorbic acid in RPMI 1640 medium and subsequently for 1 h in the presence or absence of 10 μM lipoic acid. Cells were then washed and resuspended in fresh media that included 100 μM PMC, 5 mM 3-AT, 600 μM H_2O_2. (A) Aliquots of HL-60 cells (4.8×10^6 cells/ml) were taken for HPLC analysis of ascorbate and at the indicated times after resuspension. Data are mean ± S.E., $n=6$. Content of ascorbate after cell washing taken as 100% was 3.1 ± 0.2 nmol/10^6 cells. Open circles: PMC + dehydroascorbic acid; closed circles: PMC + dehydroascorbic acid + lipoic acid. (B) Aliquots of HL-60 cells (4.8×10^6 cells/ml) were taken for HPLC analysis of PMC levels at the indicated times after resuspension. Data are mean ± S.E., $n=3$. Content of PMC after cell washing taken as 100% was 7.3 ± 0.9 nmol/10^6 cells. Closed diamonds: PMC; open squares: PMC + lipoic acid; open triangles: PMC + dehydroascorbic acid; crosses: PMC + dihydrolipoic acid + lipoic acid.

To confirm that the endogenous MPO/H_2O_2 system was responsible for generation of PMC• and that recycling occurs via endogenous ascorbate, the viability of the cells was checked by Trypan blue exclusion. Results demonstrated that about 98% of the cells were viable before addition of H_2O_2 to initiate the reaction and this did not change significantly by the end of the incubation period.

4. Discussion

Phenolic compounds, such as α-tocopherol, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), can act as potent radical scavengers by donating hydrogen to reactive radicals with consequent formation of phenoxyl radicals [20]. Similarly, endogenous, nutritional and environmental phenols, including phenolic drugs, also generate phenoxyl radicals as one-electron intermediates of their oxidation. Some phenoxyl radicals, however, may cause oxidative stress by directly attacking essential biomolecules [24] or indirectly by promoting formation of reactive oxygen species. One such pathway is via the oxidation of thiols like GSH to form thiyl (glutathionyl) radicals. Thiyl radicals may further react to generate disulfide anion-radicals (GS-$SR^{\bullet -}$) which can donate an electron to oxygen to produce superoxide, $O_2^{\bullet -}$. Superoxide and its descendant hydroxyl radical ($OH^{\cdot}$) are highly cytotoxic and only beneficial against certain tumors where an apoptosis-inducing (antitumor) impact is manifest. Other dangers lie in the inherent ability of some phenoxyl radicals to directly attack DNA and proteins. In the latter case, they may inactivate proteins (exemplified by the poisoning of DNA topoisomerase II by etoposide phenoxyl radical) or adversely affect protein stability [4]. The balance between antioxidant and prooxidant effects of phenolic compounds depends on the reactivity of phenoxyl radicals with biomolecular reductants in cells.

Previous work has identified DHLA/ascorbate as an efficient recycling system that can prevent loss of PMC by reducing PMC phenoxyl radicals back to PMC in isolated membranes fractions and lipoproteins and cell homogenates [3,13,18]. Our results, both in the model system with MPO and in live HL-60 cells with high MPO activity, clearly demonstrate that this holds true for cells (see Scheme 1). Indeed, the EPR-detected PMC phenoxyl radical signal disappeared rapidly after ascorbate was added while the ascorbate radical appeared. Only after the ascorbate radical EPR signal disappeared did the PMC radical reappear. The analytical results using HPLC were consistent with EPR measurements. PMC concentration was maintained at control levels when an ascorbate radical EPR signal could be detected by EPR spectroscopy. After the ascorbate radical EPR disappeared, HPLC measurement showed a gradual decrease of PMC concentration. The ascorbate radicals formed during this process are not sufficiently reactive to proliferate and perpetuate free radicals and other reactive oxygen species and they readily disproportionate to yield ascorbate and DHA [4]. It is clear that recycling phenoxyl radicals are effective only so long as ascorbate is available to supply reducing equivalents. Using both an isolated enzyme system (MPO/H_2O_2) and intact HL-60 cells, we observed only a very weak and long-lived ascorbate radical when MPO catalyzed oxidation of ascorbate in the absence of PMC. This is in agreement with previous reports that rate constants for ascorbate oxidation

by MPO/H_2O_2 in the absence of phenolic substrates are low ($\approx 10^4$ M^{-1} s^{-1}) [25–27]. Importantly, oxidation of ascorbate as evidenced by the EPR signals of its radicals increased proportionally to the PMC concentration as would be expected if PMC phenoxyl radicals were recycled by ascorbate. Both in the model system (MPO/H_2O_2) and in HL-60 cells with approximately the same MPO activity, increasing PMC concentration had a similar pattern of stimulated ascorbate oxidation. Slight differences (particularly at higher PMC concentrations) could be attributed to different effective concentrations of H_2O_2 due to alternative pathways of its consumption in cells (e.g., by catalase). Overall, our results clearly demonstrate that ascorbate mediates recycling of the PMC phenoxyl radical in model system and live cells without substantially competing with PMC for the activated MPO species. The present results show that additional reductants such as DHLA are able to reduce DHA to ascorbate through metabolic pathways, hence maintaining available ascorbate.

One technical problem with the strategy used in experiments with HL-60 cells is that these cells do not readily take up ascorbate [28]. This was overcome by preincubation of the cells with DHA which was subsequently reduced intracellularly to ascorbate [19] as demonstrated by EPR spectral characterization as well as by HPLC measurements. Likewise HL-60 cells do not easily take up DHLA and as such were preincubated with lipoic acid to be reduced internally [29].

Lipoic acid can be metabolically reduced by α-keto acid dehydrogenases to form DHLA, with two vicinal thiol groups. Previous reports have suggested that the protective effects of DHLA against peroxidation may involve recycling of other antioxidants such as GSH, ascorbate and vitamin E in LDL and isolated membrane fractions [15]. Although we have not addressed the role of GSH directly in the present work, literature indicates that at physiologically relevant pH, GSH does not substitute for DHLA in non-enzymatic reduction of dehydroascorbate to ascorbate [30]. However, enzymatic GSH-dependent reduction of dehydroascorbate to ascorbate has been reported [31,32]. It is likely that the thioredoxin/thioredoxin reductase system may play the same role as DHLA in recycling ascorbate and vitamin E [33]. This is why the proposed DHLA recycling system may be important to prolong the effects of ascorbate. Our results demonstrated that this recycling cascade DHLA → ascorbate → vitamin E functions in cells. It is noteworthy that DHLA alone had no direct effect on PMC phenoxyl radicals. Thus, when DHLA was added along with PMC, no significant change was observed in the PMC radical signal. Some protective effect of DHLA on loss of PMC was found by HPLC analysis but there was apparently no quenching of the oxidation of PMC. In contrast, when ascorbate was combined with DHLA, PMC radicals were quenched and PMC concentration was maintained for a significantly longer time than in the absence of ascorbate. It was clear that this recycling effect of DHLA on PMC relied on ascorbate recycling. EPR measurements showed that ascorbate radicals were sustained for longer periods when ascorbate was present in combination with DHLA. In addition, HPLC measurement of ascorbate showed a protective effect of DHLA on ascorbate oxidation. These results clearly demonstrate that thiols (DHLA) are not directly oxidized by PMC phenoxyl radicals. The importance of this is emphasized by the known ability of some phenolics to induce "futile" thiol pumping [34] triggering thiyl radical as well as superoxide radical production. Obviously, such phenolic compounds, in contrast to vitamin E and its homologues such as PMC, do not qualify to fulfill any antioxidant protective role as their phenoxyl radicals propagate generation of reactive radicals as opposed to their quenching by effective antioxidants.

In cells containing peroxidase capacity such as CD34+ myeloid progenitor cells [35,36] the phenolic-rich milieu provides ample opportunity for oxidative radical production (of flavonoids, anthocyanins, tyrosine, steroids, etc.). These cells are also exposed to numerous exogenous phenolic compounds, environmental chemicals and drugs. In addition, cytochrome P450-catalyzed hydroxylation reactions produce numerous metabolites that may be converted into cytotoxic phenoxyl radicals. In accordance with their pro-oxidant and antioxidant potentials, these phenolics have a dual role in cells. The pathogenesis and treatment of a variety of disorders exemplify this dichotomy. For example, etoposide, the second most widely used antitumor drug, is closely associated with treatment-related leukemia (t-AML) that may derive from MPO-containing CD34+ myeloid progenitors [3]. Promyeloid HL-60 cells (containing MPO and H_2O_2) have been demonstrated to generate reactive VP-16 phenoxyl radicals that can readily attack thiols [24,37]. Furthermore, peroxidase activation of *o*-phenylphenol polycarbonylbiphenyls may play a role in the carcinogenic effects of these compounds via DNA adduct formation in some cell lines [38]. Phenolic estrogens protect against atherogenesis by inhibiting sub-endothelial LDL oxidation [39], yet estrogen-derived semiquinones are implicated in tumor growth [40]. It has been suggested that the tendency of any species towards pro-/anti-oxidant behavior depends on factors such as metal-reducing potential, chelating behavior, pH and solubility characteristics [4,41].

We propose an antioxidant intervention that may provide a phenoxyl radical bypass mechanism to cells when these species pose a threat. Indeed, PMC, being a substrate for oxidative enzymes such as MPO, yields low-reactive phenoxyl radicals that do not result in significant oxidation of intracellular thiols or carotenoids and do not peroxidize membrane phospholipids [17]. Thus vitamin E homologues including PMC can be used as nontoxic competitive MPO substrates to abolish or attenuate toxic effects of compounds whose MPO-catalyzed metabolism generates reactive phenoxyl radicals. One such example is the potential prevention of cytotoxic/genotoxic effects of etoposide [3]. A very important facet of this proposed therapy is the extension

of the lifetime of PMC through its ascorbate/DHLA-supported recycling.

This antioxidant recycling system demonstrated in live HL-60 cells is probably not exclusive to PMC, but is likely to take place with other phenolic compounds. Thiols, ascorbate and other reductants can reduce the phenoxyl radicals produced, regenerating the parent phenolic compound [3,5,10,42,43]. High concentrations of phenols and ascorbate are found in a variety of berry fruits [44] and the present study may explain the overall antioxidant effects in these natural products.

A future prospect offered by these results is the possibility of counteracting oxidative stress in cells by stimulating recycling of phenoxyl radicals enzymatically generated from antioxidants. Many cancers originate in peroxidase-rich target tissues by mechanisms not fully understood [3,17]. These mechanisms do, however, unequivocally involve oxidative stress so treatments incorporating metabolically disposable antioxidants with low pro-oxidant profiles have an enormous potential as durable chemopreventative therapies.

Acknowledgements

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