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Advances in Brief

Increased Generation of Lipid-derived and Ascorbate Free Radicals by L1210 Cells Exposed to the Ether Lipid Edelfosine[1]

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Abstract

Using the spin trap α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone, we have detected a lipid-derived carbon-centered free radical generated from intact L1210 lymphoblastic leukemia cells that were exposed to 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (edelfosine or ET-18-OCH_3) and oxidative stress. The spectral characteristics, including hyperfine splitting constants of a^N = 15.61G and a^H = 2.65G, were consistent with the spin trapping of an alkyl radical. Radical detection required iron and prior enrichment of cellular components with the polyunsaturated fatty acid docosahexaenoic acid; unmodified cells failed to generate detectable free radical. Ascorbate further enhanced radical generation. The detection of lipid-derived free radicals when intact cells are exposed to edelfosine provides further evidence that oxidative stress may play an important role in the cytotoxic mechanism of this class of anticancer drug.

Introduction

The ether lipids are membrane-active anticancer drugs with a wide spectrum of antineoplastic and biological activity. Their mechanism of action remains unknown. We have recently reported that these agents enhance iron-induced lipid peroxidation of neoplastic cells (1). We have now detected a lipid-derived free radical from docosahexaenoic acid-enriched leukemia cells during oxidative stress and shown that edelfosine enhances the formation of this radical species.

Materials and Methods

Fatty Acid Modification. L1210 cells were grown for 48 h in RPMI 1640 containing 5% fetal bovine serum and 32 μM 22:6[2] (Nu Chek Prep, Inc., Elysian, MN) (2). We have shown previously that supplementation of growth media with 22:6 results in cells that contain 23–37% of 22:6 in cellular phospholipids (1, 3, 4). For comparison, unmodified cells contain <1% 22:6 (1, 5).

Ascorbate and Lipid-derived Radicals from Intact Cells. Fatty acid-modified L1210 cells were washed and placed in 0.9% NaCl. In initial studies, 10 mM POBN (Sigma Chemical Co., St. Louis, MO), 20 μM $FeSO_4 \cdot 7H_2O$ (Fisher Scientific Co., Fair Lawn, NJ), 100 μM ascorbic acid (Mallinckrodt, Inc., Paris, KY) and edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine or ET-18-OCH_3; Medmark Pharma GmbH, Grünwald, Germany; kindly supplied by Dr. R. Nordström) were added to the air-saturated cell suspension, in that order, and placed into an EPR quartz flat cell at room temperature and scanned by EPR. Time course studies were initiated by adding to L1210 cell suspensions, 50 mM POBN, 100 μM ascorbic acid, and then 20 μM Fe^{2+}. After monitoring by EPR for 190 s, edelfosine was added and scanning was continued. For studies of the general cellular oxidative state, as estimated by the ascorbate free radical signal intensity, no POBN was added. Quantitation of ascorbate radical signal intensities from EPR scans were determined as described using 3-carboxyproxyl as a standard (6).

Electron Paramagnetic Resonance. Spectra were measured using a Bruker ESP 300 spectrometer equipped with a TM_{110} cavity and operating at 9.77 GHz microwave frequency and 100 kHz modulation frequency.

Results

Spin Trapping of Lipid-derived POBN-Spin Adducts from Cells. When L1210 cells enriched with 22:6 were incubated in the presence of 10 mM POBN, no spin adducts were detected (Fig. 1*A*). Likewise, neither 22:6-enriched L1210 cells treated with 40 μM edelfosine and 10 mM POBN without any oxidant cofactors (Fig. 1*B*) nor unmodified L1210 cells treated with edelfosine and cofactors (not shown) produced detectable POBN spin adducts or other EPR detectable radical species. Repeated experiments with higher concentrations of edelfosine failed to produce any detectable POBN spin adducts. Therefore, edelfosine does not produce EPR-detectable free radicals in the absence of oxidative cofactors or in cells with low polyunsaturated fatty acid content.

22:6-enriched cells incubated with 100 μM ascorbic acid produced a weak ascorbate radical signal (Fig. 1*C*), which dissipated with time. When 20 μM Fe^{2+} was added to POBN-treated cells, a carbon-centered spin adduct was detected (Fig. 1*D*). This adduct spectrum was stable in intensity for at least 30 min (data not shown). Cells treated with iron and ascorbic acid (Fig. 1*E*) produced a carbon-centered POBN spin adduct similar to the one found in cells treated with iron alone (Fig. 1*D*), which is superimposed on an ascorbate radical signal. This POBN-spin adduct EPR signal was more intense than those from cells treated with iron alone. The ascorbate radical spectrum, which was high in intensity at the early time points of 2–5 min, dissipated with time, and became undetectable at 30 min (data not shown). In general there was a inverse relationship over time between the intensity of the POBN-spin adduct and that of the ascorbate radical.

The addition of 40 μM edelfosine to 22:6-enriched cells with iron and ascorbic acid also caused the appearance of both the carbon-centered spin adduct and the ascorbate radical (Fig. 1*F*). The carbon-centered spin adduct and spectral characteristics of the ascorbate radical were similar in configuration to those observed in cells treated with iron and ascorbic acid; however, in all experiments, cells treated with edelfosine appeared to have higher carbon-centered radical intensities than those treated with $FeSO_4$ and ascorbic acid, alone or in combination.

Kinetics of Lipid-derived Radical Formation in Whole Cells: Augmentation by Edelfosine. In internally controlled EPR experiments, 22:6-enriched L1210 cells were incubated with 50 mM POBN spin trap, 20 μM Fe^{2+}, and 100 μM ascorbate and monitored by EPR (Fig. 2). At 190 s after the initiation of the experiment, varying concentrations of edelfosine were added to the cell suspension. The addition of edelfosine resulted in intensification of the POBN-adduct formation in a concentration-dependent manner beginning 20–60 s after addition of the drug. Analysis of covariance comparing controls

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[2] The abbreviations used are: 22:6, docosahexaenoic acid; POBN, α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone; EPR, electron paramagnetic resonance.

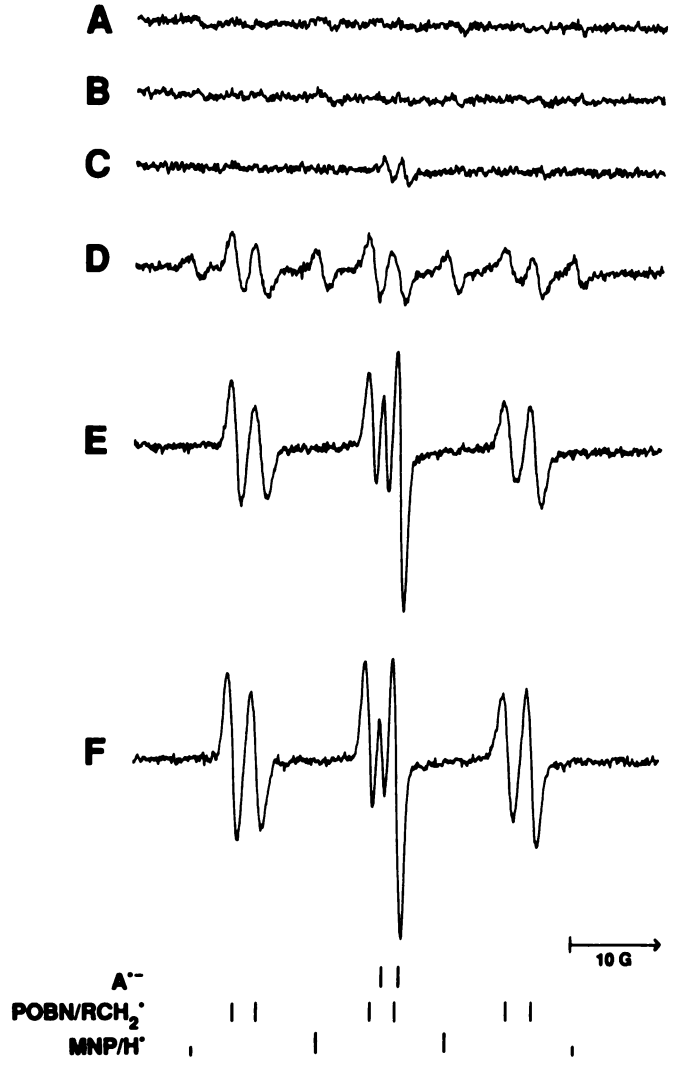

Fig. 1. EPR spectra of POBN-spin adducts from whole cell incubations during peroxidation. 22:6-enriched L1210 cells (1×10^7/ml) were subjected to iron-induced oxidative stress at room temperature in air-saturated 0.9% NaCl (4–8 min) and then analyzed by EPR. All incubations were done in the presence of 10 mM POBN. $A^{\cdot -}$, ascorbate radical; *POBN/RCH₂·*, POBN adduct of an alkyl radical such as ethyl, pentyl, or alkyl; *MNP/H·*, 2-methyl-2-nitroso-propane/hydrogen atom spin adduct. *A*, 22:6-enriched L1210 cells. *B*, 22:6-enriched L1210 cells with 40 μM edelfosine. *C*, 22:6-enriched L1210 cells with 100 μM ascorbic acid. Only the ascorbate radical is detected, a^H = 1.81G. *D*, 22:6-enriched cells with 20 μM $FeSO_4$, a^N = 15.58G, a^H = 2.73G. Also present is an additional POBN-derived radical signal. This artifactual signal, 2-methyl-2-nitrosopropane/H·, is likely derived from decomposition of the POBN spin trap (7), since this spectral pattern of four peaks (1:2:2:1 intensity ratio) is not characteristic of POBN spin adducts. *E*, 22:6-enriched L1210 cells with 20 μM $FeSO_4$ and 100 μM ascorbic acid, a^N = 15.52G, a^H = 2.75G. The ascorbate radical doublet is also present. *F*, 22:6-enriched L1210 cells with 40 μM edelfosine, 20 μM $FeSO_4$, and 100 μM ascorbic acid, a^N = 15.61G, a^H = 2.65G. The EPR spectrometer settings were: receiver gain 5.0×10^5; modulation amplitude 1.0G; scan rate 60 G/168 s; time constant 164 ms; microwave power 40 mW at a frequency of 9.77 GHz.

without edelfosine to experimentals (5–80 μM edelfosine) were significantly different at $P < 0.05$.

Effect of Edelfosine on Ascorbate Radical Formation during Peroxidation in Whole Cells. Ascorbate radical EPR signal intensity can be used as a measure of oxidative flux (6). The introduction of edelfosine to 22:6-enriched cell samples, previously incubated with iron and ascorbic acid to initiate peroxidation, caused increased ascorbate radical intensity in an edelfosine concentration-dependent manner at concentrations above 20 μM (Fig. 3). Ascorbate radical intensity peaked 40–60 s after the addition of ET-18–OCH_3. With 30 μM edelfosine there appeared to be a delay in the peak ascorbate radical intensity until 110–130 s after the introduction of the drug.

Discussion

Our unique EPR techniques allow real-time detection of free radical generation from live cells, permitting us to monitor the effects of therapeutic maneuvers on oxidative events. Enhanced radical generation in cells in the presence of ether lipid was particularly unexpected since the metabolism of this class of drug is not known to involve the generation of free radicals. The spin-trapped radical detected from

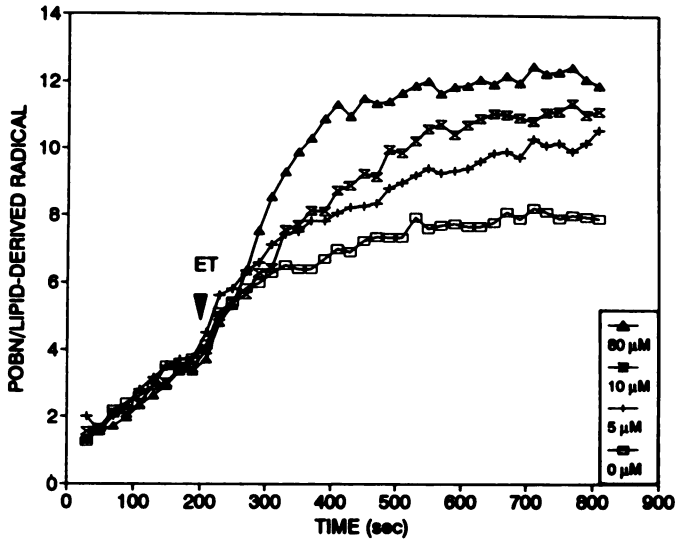

Fig. 2. Effect of edelfosine concentration on POBN spin adduct formation from whole cell incubations during oxidative stress. 22:6-enriched L1210 cells ($5.0\ 10^6$/ml) were incubated at room temperature in air-saturated 0.9% NaCl in water with 20 μM Fe^{2+} and 100 μM ascorbic acid in the presence of 50 mM POBN for a brief monitoring period by EPR. Then, edelfosine (*ET*) at various concentrations was added (*arrowhead*) and monitored by EPR. The curves were significantly different from controls containing iron and ascorbic acid alone at $P < 0.05$ by analysis of covariance. EPR settings were: receiver gain 5×10^5; modulation amplitude 0.63 G; scan rate 50 G/21 s; time constant 20.5 ms; microwave power, 40 mW at a frequency of 9.78 GHz.

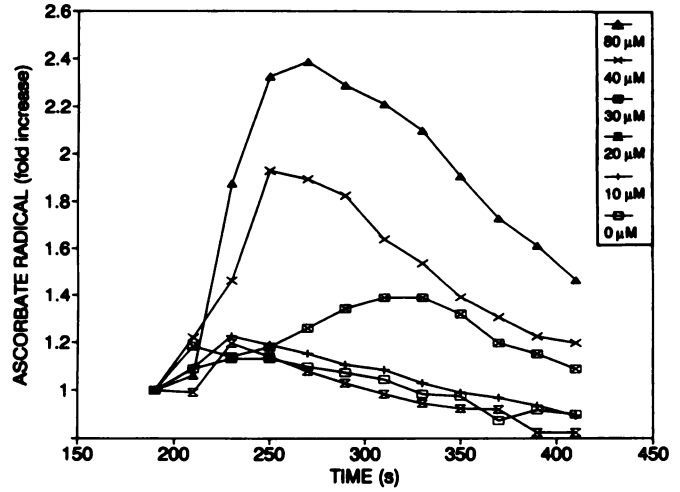

Fig. 3. Effect of edelfosine concentration on ascorbate radical formation from whole cell incubations during peroxidation. Experiments were carried out as described in the legend of Fig. 2 but without the presence of spin trap. The iron and ascorbic acid were added to the incubation and the edelfosine was added just after the 190-s scan. Times correspond to those of spin trap experiments in Fig. 2 in order to allow comparison of the kinetics of the generation of the two types of radicals. The peak ascorbate radical intensity corresponds to 82 nM. EPR settings were: receiver gain 6.3×10^5; modulation amplitude 0.63 G; scan rate 6 G/21 s; time constant 328 ms; microwave power 40 mW at a frequency of 9.79 GHz.

intact cells has the spectral characteristics of an alkyl radical adduct of POBN, probably ethyl, pentyl, or pentenyl radical adduct. This is the same radical observed from iron-induced oxidative stress in intact cells as recently reported by North *et al.* (8). This alkyl radical results from β-scission of lipid alkoxyl radicals formed by the reaction of ferrous iron and lipid hydroperoxides (9). Radical production depends upon the presence of iron; ether lipid appears to augment iron-induced free radical generation. This could occur by an effect of the anticancer drug on oxidant entry into the lipid bilayer, translocation of key enzymes or cofactors of the oxidative process, or changes in membrane physical properties that increase oxidative susceptibility of cells.

Lipid peroxidation has three components, initiation, propagation, and termination. The association of ether lipid with the membranes of cells could affect any of these. For example, it could increase the rate of extraction of a hydrogen atom from an unsaturated fatty acid, thereby enhancing initiation. Alternatively, the drug could decrease the rate of termination, thereby increasing the propagation chain length resulting in an overall amplification in lipid peroxidation. In either case, peroxyl, alkoxyl, and alkyl radicals are formed, and peroxidation of cell membranes can be detected as measured by other techniques (1). Our observations on the enhanced generation of ascorbate free radical, which reflects a heightened cellular oxidative state (1, 6), do not allow differentiation of these possibilities but are consistent with a role of oxidative stress in the action of this drug. The cytotoxicity of edelfosine could result entirely from oxidative events involving lipid peroxidation.

The generation of a lipid radical as a result of the ether lipid drug is a rapid process occurring in minutes; in contrast, cytotoxicity is delayed. This lag time could result from a series of intermediate steps such as propagation of peroxidation or others which are not yet identified. A similar delay occurs with radiation cytotoxicity in that the initial free radical generation event and survival kinetics are staggered.

These observations are important because they provide a possible explanation for the cytotoxicity of the ether lipids. Other hypotheses have been suggested, such as an effect of these drugs on protein kinase C (10), inhibition of phospholipase C (11), inhibition of growth factor-dependent inositol phosphate Ca^{2+} signaling (12), and induction of cellular differentiation (13). It seems likely that iron-induced lipid peroxidation and free radical generation plays some role since it is an intense, easily detected process that occurs in the presence of physiological amounts of iron and is enhanced by physiological amounts of ascorbate. Concurrent free radical or peroxidative damage to nuclear DNA or cellular proteins may also play a part. We have recently reported that 22:6-enriched cells are more sensitive to an ether lipid drug (14). The possibility of increasing anticancer efficacy by prior enrichment of tumor cells with polyunsaturated fatty acids could be of use in the treatment of experimental or clinical cancer with this class of drug.

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