Cell fatty acid composition affects free radical formation during lipid peroxidation

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North, James A., Arthur A. Spector, and Garry R. Buettner. Cell fatty acid composition affects free radical formation during lipid peroxidation. Am. J. Physiol. 267 (Cell Physiol. 36): C177–C188, 1994.—Lipid-derived free radicals generated from intact human U937 monocytes exposed to iron-induced oxidative stress were detected by electron paramagnetic resonance (EPR) with the spin trap $\alpha$-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN). Lipid radical formation was enhanced when the cells were enriched with n-3 or n-6 polyunsaturated fatty acids. Computer simulation indicated that at least two POBN spin adducts were formed, having spectral characteristics consistent with carbon-centered radicals ($a^N = 15.9$ G and $a^H = 2.6$ G; $a^N = 15.1$ G and $a^H = 2.8$ G). These alkyl radicals are probably formed by $\beta$-scission of alkoxyl radicals. POBN spin adduct formation correlated with ethane generation. Addition of ascorbate to the assay medium greatly increased the radical signal intensity. Although radical generation was cell dependent and POBN spin adducts were observed in cell homogenates, the adducts formed by the intact cells were detected only in the extracellular medium. These findings indicate that the extent of lipid radical formation in response to oxidative stress can be influenced by changes in the polyunsaturated fatty acid composition of the cell lipids and suggest the possibility that carbon-centered lipid radicals may interact with extracellular structures.

FREE RADICALS are generated in a number of metabolic reactions, and lipids containing polyunsaturated fatty acids in cell membranes and lipoproteins are targets of free radical-mediated oxidation (17). This process of lipid peroxidation consists of three components: initiation in which free radicals are formed, propagation of the radical chain reactions, and termination (6, 25, 28). Iron or other catalytic metals usually are required to initiate lipid peroxidation (25), and the free radicals generated, such as a lipid-derived carbon-centered radical (L·), lipid peroxyl radical (LOO·), and lipid alkoxyl radical (LO·), propagate the chain reactions (28). Termination of lipid peroxidation results when the free radicals in the chain propagation step react with other free radicals or antioxidants to form nonradical short-chain hydrocarbon compounds.

We recently reported the electron paramagnetic resonance (EPR) detection of lipid-derived carbon centered free radical $\alpha$-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN) spin adducts from a suspension of intact human U937 monocytic leukemia cells exposed to oxidant stress (26). This process was initiated by exposure of the U937 cells to ferrous ions; free radicals were detected by EPR spin trapping. The cell suspensions that generated easily detectable amounts of spin adducts were supplemented with substantial quantities of docosahexaenoic acid [22:6(n-3), DHA], a polyunsaturated fatty acid containing six double bonds and five methylene carbons that is highly susceptible to lipid peroxidation.

The purpose of the present study was to characterize the conditions necessary for lipid radical formation in intact U937 cells and determine whether this process requires the presence of large amounts of DHA in the cell lipids. During the course of this work, we found that the spin adduct formed by the U937 cells was present in the extracellular fluid. This finding suggests that some lipid radicals either are formed near the cell surface or can otherwise gain access to the extracellular medium, where they might be in a position to initiate oxidative reactions in adjacent extracellular structures.

METHODS

Materials. POBN and iminodiacetic acid chelating resin were purchased from Sigma (St. Louis, MO). 3- Carboxy-proxy1 was obtained from Aldrich (Milwaukee, WI). Fatty acids purchased from Nu Chek Prep (Elysian, MN) included DHA [22:6(n-3)], eicosapentaenoic acid [20:5(n-3)], arachidonic acid [20:4(n-6)], eicosatrienoic acid [20:3(n-3)], linoleic acid [18:3(n-6)], linoleic acid [18:2(n-6)], and oleic acid [18:1(n-9)]. 1-[1-14C]DHA (40–60 mCi/mmole) was purchased from Du Pont-New England Nuclear Research (Boston, MA). A 0.25 M POBN aqueous stock solution was prepared immediately before use and kept on ice during the experiment.

Cell culture. The human U937 monocytic leukemia cell line was grown in a humidified atmosphere of 95% air-5% CO2 at 37°C in RPMI 1640 medium containing 5% fetal bovine serum, 15 mM N2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 2 mM L-glutamine (18). The cells were maintained as an exponentially growing culture by passage every 2–3 days. Cell lipids were modified by the addition of 20 μM fatty acid to the growth medium for 2 days (26). Before the experiment, the cells were washed twice with a phosphate-buffered salt solution (PBS) containing 2.7 mM KH2PO4, 136.8 mM NaCl, and 8.1 mM Na2HPO4 (pH 7.4) to remove any remaining fatty acid-supplemented growth medium. Cells were then resuspended in the PBS at a concentration of ~4 x 10⁶ cells/ml. PBS was treated with chelating resin to remove adventitious catalytic metals using the batch method (5), the absence of catalytic metals was verified with ascorbate (5). Cell protein content was assayed by a modification of the Lowry method in which sodium dodecyl sulfate was added (23). Cell cultures were tested and found to be negative for mycoplasma.

Fatty acid composition. Fatty acid composition was determined by gas-liquid chromatography (GLC) (16). Briefly, washed cells were extracted with CHCl3-CH3OH (2:1, vol/vol) (91), and the lipids were saponified for 45 min at 70°C in 10 N KOH-95% ethanol (0.5:10, vol/vol) (14). A known amount of heptadecanoic acid methyl ester (17:0) was added as an internal standard. The fatty acids then were methylated for 10 min at 95°C with 12% BF3 (16) and separated by GLC using a
1.8-m column packed with 10% SP2330 on 100–120 mesh Chromosorb Gaschrome WAW. The resultant peaks were identified by comparing the retention times with those of known fatty acid methyl ester standards, and peak areas were determined by automatic integration. Statistical analysis was done using the t test.

**Incubations.** Unless noted otherwise, 0.5 ml PBS containing 4 × 10⁶ cells/ml was used in all incubations. Immediately before placement in the EPR spectrometer cavity, 10 mM (final concn) POBN was added to the cell suspension. Ferrous iron (10 μl) from a stock solution of 5 mM FeSO₄ · H₂O in 10 ml H₂O, containing 50 μl concentrated H₂SO₄ to stabilize the iron as Fe²⁺, was added next. The contents of the tube were gently mixed by vortexing and then transferred to an EPR quartz flat cell. In additional experiments, either 10 mM N-tert-butylnaphthylamine (POBN) or 2-methyl-2-nitroso propane (MNP) was substituted for POBN as the spin trap.

Disruption of cells was done by nitrogen cavitation as described previously (21), except that the disruption buffer consisted of PBS alone. In one experiment, the homogenate was centrifuged at 600 g to remove nuclei and unbroken cells. The resulting supernatant material was mixed with 10 mM POBN and 100 μM FeSO₄, and EPR spectra were collected. In another experiment, the 600-g supernatant material was centrifuged at 10,000 g to remove mitochondria, and the microsome fraction was then isolated by sedimentation for 1 h at 100,000 g. After washing, the fatty acid composition of the isolated microsomes was assayed by GLC (14, 26).

**Incorporation and release of [1-14C]DHA.** To determine the distribution of newly incorporated DHA in the cell lipids, the U937 cells were incubated with 5 μM [1-14C]DHA, washed, and extracted with 20 ml of the CHCl₃-CH₃OH mixture. After the CHCl₃ phase was isolated, the radioactivity was measured in an aliquot by liquid scintillation spectrometry, and the distribution of the radioactivity among the major lipid classes was determined in the remainder of the CHCl₃ extract after separation by thin-layer chromatography (TLC) (16). In another experiment, the cells were similarly labeled with [1-14C]DHA, and after washing, the cell suspension was divided into four equal portions. Two of these were incubated in PBS, and the other two in PBS containing 100 μM FeSO₄, and EPR spectra were collected. In another experiment, the 600-g supernatant material was centrifuged at 10,000 g to remove mitochondria, and the microsome fraction was then isolated by sedimentation for 1 h at 100,000 g. After washing, the fatty acid composition of the isolated microsomes was assayed by GLC (14, 26).

**EPR measurements.** EPR spectra were obtained using a Bruker ESP-300 spectrometer operating at 9.79 GHz with 100-kHz modulation frequency. Each sample, contained in a quartz flat cell, was centered in a TM10 cavity. All EPR measurements were made using air-saturated solutions at room temperature. The EPR spectrometer settings were as follows: microwave power, 40 mW (7); modulation amplitude, 1.05 G; time constant, 1.3 s; scan rate, 60 G/335 s; receiver gain, 1.0. Quantitation of spectra was done using 3-carboxy-proxyl as the radical standard (13). Accurate determinations of POBN spin adduct concentrations by standard double integration in samples with very low signal-to-background ratio could not always be reliably achieved using the Bruker ESP-300 software package. In these cases peak heights were used; values for the height of the first midfield peak and the trough of the second midfield peak were measured. Those spectra having a high signal-to-background ratio were analyzed by both double integration and measurement of midfield peak ranges. Analysis by either method yielded similar radical concentrations. Computer simulation of EPR spectra was performed utilizing programs available from the National Institute of Environmental Health Sciences (12).

**Results**

**EPR spin-trapping studies.** The spectral intensity of POBN spin adducts (a⁵N − 15.61 ± 0.04 G and a⁵H = 2.64 ± 0.03 G), generated after iron-mediated oxidative stress, was determined relative to the amount of supplemental DHA present in the growth medium of the U937 cells (Fig. 1). In these and all other studies in which free radicals were measured, the cells were removed from the medium containing the fatty acid supplement and washed before they were incubated with POBN and ferrous iron. Therefore no lipid supplement was contained in the medium when the EPR measurements were made. The intensity of the carbon-centered radical adduct measured by EPR increased as the concentration of DHA to which the cells had been exposed in the growth medium was raised. With the instrument settings used, this adduct was not discernibly detected when DHA-supplemented cells were incubated with POBN alone (Fig. LA). It was also not discernible when cells grown either without supplemental fatty acid (Fig. 1B) or with 2.5 μM DHA (Fig. 1C) were incubated with POBN and ferrous iron. However, the POBN spin adduct was clearly detected when the growth medium was supplemented with 5 μM DHA (Fig. 1D), and the signal intensity became more pronounced as the supplemental DHA concentration was increased further (Fig. 1, E–H). On the basis of the work using cell-free incubations with purified polyunsaturated fatty acids and lipoxygenases (9, 20), we have assigned the spectra to an alkyl radical adduct of POBN, such as an ethyl, pentyl, or pentenyl radical.

Quantitation of the spectra illustrated in Fig. 1 using a 3-carboxy-proxyl standard shows that the amount of radical generated from U937 cells exposed to oxidative stress increased sharply when the supplemental DHA concentration to which the cells had been exposed in the growth medium was increased above 10 μM (Fig. 2).
Because there is considerable scatter in the data obtained from the cells grown in the presence of 40 μM DHA, it appears that radical formation may plateau at supplemental concentrations between 30 and 40 μM DHA.

Ethane production. The relationship between POBN radical adduct formation and ethane generation by the U937 cells was compared. When the cells were grown in medium supplemented with 10 μM DHA, radicals were trapped and ethane was generated (Table 1). In corresponding cells grown in medium supplemented with oleic acid, lipid-derived radical formation was too low to be detected, and very little ethane was produced.

Enrichment of cell lipids with DHA. The fatty acid composition of cells grown in media supplemented with DHA was determined by GLC. The percentage of total fatty acid present as DHA increased as the amount of DHA supplementation was raised, reaching a plateau at 40 μM added DHA (Fig. 3). DHA accounted for 2.5% of the total fatty acid in the fetal bovine serum present in the growth medium, and this probably accounts for the small amount of DHA present in the cells grown in the medium that did not contain any supplemental fatty acid.

The fatty acid composition of the isolated microsomes was also determined after growth of the U937 cells in medium supplemented with either 20 μM DHA or oleic acid. As a control, microsomes from cells grown in medium without a fatty acid supplement also were isolated and assayed. Compared with the microsomes from the unsupplemented cells, a 10-fold increase occurred in the relative amount of 22:6 in the cells supplemented with DHA, and there was a doubling in the relative content of polyunsaturated fatty acid (Table 2). Likewise, there was a 30% increase in the relative amount of 18:1 in the microsomes from the cells supplemented with oleic acid. Therefore fatty acid compositional changes similar to those observed in the intact cells also occurred in the membranes that comprise the microsomal fraction.

The distribution of the incorporated DHA among the cell lipids was also measured after supplementation. After incubation for 24 h with 5 μM DHA containing [1-14C]DHA, the cells were washed and the radioactivity in the extracted lipids was determined after separation by TLC. The radioactivity was distributed primarily among three lipid fractions: 35% in phosphatidylinoline, 18% in phosphatidylethanolamine, and 30% in triglycerides (data not shown).

An experiment was also done to determine whether lipids containing DHA might be discharged from the cells as a result of exposure to FeSO4. After labeling with the [1-14C]DHA and washing, portions of the labeled cell suspension were incubated for 15 min with PBS containing 100 μM FeSO4 or PBS alone as a control. In both cases, 5.2–5.4% of the cell radioactivity was recovered in the medium at the end of the 15-min incubation. Release of a small amount of radioactive lipids during incubation of cells containing labeled DHA has been observed previously (16). The present findings demonstrate that the FeSO4-mediated oxidation did not cause a measurable increase in this release process. Because [1-14C]DHA was used to label the cells, however, this assay is not able to detect the release of oxidation products that do not contain the carboxyl carbon of the added DHA.

Factors affecting radical formation. Trapping of the carbon-centered radical was dependent on the order of addition of compounds into the reaction mixture. When ferrous iron was added before POBN, no lipid-derived radical was detected; the POBN had to be added before FeSO4 to trap a carbon-centered radical. Adding 1.8 mM final concentration sulfuric acid, which was equal to that used to prepare the FeSO4 stock solution, to DHA-enriched cells in the presence of POBN lowered the pH of the incubation mixture to 7.0 but did not generate a radical signal.

In most of the experiments, the POBN concentration was 10 mM. To ascertain whether this amount was optimal, cells were incubated with various amounts of POBN and after addition of 100 μM FeSO4, EPR spectra were obtained. The radical adduct peak heights were determined, and the amount of radical formation was calculated using the 3-carboxy-proxy1 standard. Increas-
Fig. 2. Effect of DHA concentration on quantity of POBN spin adducts generated. Spin adducts formed when 2 × 10⁶ U937 cells, which had been previously enriched with DHA for 48 h, were incubated with 10 mM POBN and 100 μM FeSO₄ in a total volume of 0.5 ml. Each point represents results obtained from at least 3 separate experiments ± SE.

ing the POBN concentration from 0 to 20 mM resulted in larger amounts of radical adduct formation (Fig. 4). At POBN concentrations <2.5 mM, however, the amount of radical adducts formed was equal to or less than the background values at the spectrometer settings used. Although the 80 mM POBN produced a slightly larger amount of trapped radicals than 10-40 mM POBN, breakdown of the spin trap occurred in the presence of FeSO₄ when the POBN concentration exceeded 40 mM. This produced significant artifactual species and interfered with the lipid-radical spectral analysis. Furthermore, trypan blue exclusion studies indicated that POBN concentrations above 40 mM reduced U937 cell viability.

Table 1. Ethane production and radical adduct generation in DHA supplemented U937 cells

<table>
<thead>
<tr>
<th>Fatty Acid Supplement</th>
<th>Peroxidation Products, pmol/(4 × 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethane</td>
</tr>
<tr>
<td>Docosahexaenoic</td>
<td>14.9 ± 1.7</td>
</tr>
<tr>
<td>Oleic</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Cells were grown for 2 days with 10 μM supplemental fatty acid, washed three times with phosphate buffered saline, and resuspended to 10⁶ cells/ml. After exposure to 200 μM FeSO₄, ethane production was measured from headspace of a 10-ml syringe injected into gas chromatograph. Radical adduct generation was quantitated using 4 × 10⁶ cells/ml exposed to FeSO₄ in the presence of 10 mM a-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN). Data for cells enriched with docosahexaenoic acid (DHA) represent mean ± SE of 3 individual determinations, whereas data for cells enriched with oleic acid are average of 2 determinations. Radical adducts were not measurable (NM) at instrument settings used with cells enriched with oleic acid.

Fig. 3. Correlation between DHA concentration in growth medium and its content in cell lipids. U937 cells were grown for 48 h in medium supplemented with various concentrations of DHA. Cells were washed 3 times, lipids were extracted with CH₂Cl₂-CH₃OH (2:1, vol/vol), and lipid extracts were subjected to alkaline hydrolysis. Fatty acids contained in saponifiable fraction were methylated, and methyl esters were separated by gas-liquid chromatography. Each point represents results obtained from at least 3 separate experiments ± SE, except for cells grown in absence of added DHA for which result is average of 2 individual samples from 2 different experiments.
Table 2. Growth of U937 cells in presence of fatty acid supplements affects fatty acid composition of microsomes

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>No fatty acid supplement ((n = 3))</th>
<th>Oleic acid supplement ((n = 3))</th>
<th>DHA supplement ((n = 3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>2.0 \pm 0.4</td>
<td>0.9 \pm 0.1</td>
<td>2.3 \pm 0.6</td>
</tr>
<tr>
<td>16:0</td>
<td>15.3 \pm 1.8</td>
<td>13.9 \pm 0.4</td>
<td>17.3 \pm 4.7</td>
</tr>
<tr>
<td>16:1</td>
<td>5.2 \pm 0.8</td>
<td>2.8 \pm 0.2</td>
<td>3.3 \pm 0.2</td>
</tr>
<tr>
<td>18:0</td>
<td>15.4 \pm 1.5</td>
<td>13.5 \pm 0.9</td>
<td>9.7 \pm 2.2</td>
</tr>
<tr>
<td>18:1</td>
<td>23.7 \pm 2.4</td>
<td>31.1 \pm 7.1</td>
<td>11.2 \pm 3.6</td>
</tr>
<tr>
<td>18:2</td>
<td>1.3 \pm 0.3</td>
<td>1.1 \pm 0.4</td>
<td>1.1 \pm 0.2</td>
</tr>
<tr>
<td>18:3</td>
<td>1.9 \pm 0.1</td>
<td>3.8 \pm 0.7</td>
<td>1.2 \pm 0.1</td>
</tr>
<tr>
<td>20:3</td>
<td>1.6 \pm 0.2</td>
<td>1.5 \pm 0.1</td>
<td>1.2 \pm 0.4</td>
</tr>
<tr>
<td>20:4</td>
<td>7.6 \pm 0.3</td>
<td>5.0 \pm 0.3</td>
<td>4.5 \pm 0.2</td>
</tr>
<tr>
<td>20:5</td>
<td>2.5 \pm 1.0</td>
<td>1.1 \pm 0.8</td>
<td>4.2 \pm 1.3</td>
</tr>
<tr>
<td>22:4</td>
<td>2.5 \pm 0.3</td>
<td>2.7 \pm 0.3</td>
<td>1.6 \pm 0.9</td>
</tr>
<tr>
<td>22:5</td>
<td>3.4 \pm 0.3</td>
<td>2.5 \pm 0.5</td>
<td>4.0 \pm 2.6</td>
</tr>
<tr>
<td>22:6</td>
<td>2.8 \pm 0.7</td>
<td>0.6 \pm 0.6</td>
<td>29.5 \pm 0.9</td>
</tr>
<tr>
<td>Saturated</td>
<td>34 \pm 1</td>
<td>30 \pm 6</td>
<td>30 \pm 7</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>30 \pm 3</td>
<td>35 \pm 7</td>
<td>15 \pm 4</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>24 \pm 1</td>
<td>18 \pm 1</td>
<td>47 \pm 5</td>
</tr>
<tr>
<td>Unsaturation index</td>
<td>1.29</td>
<td>1.05</td>
<td>2.66</td>
</tr>
<tr>
<td>Chain-length index</td>
<td>1.67</td>
<td>14.8</td>
<td>17.6</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE. U937 cells were grown for 48 h in a medium containing 5% fetal bovine serum and no fatty acid supplement, 20 \(\mu\)M supplemental oleic acid, or 20 \(\mu\)M supplemental DHA. After washing and resuspension, cells were homogenized, and a microsomal fraction isolated by sedimentation for 1 h between 10,000 and 100,000 g. Lipids were extracted with \(\text{CHCl}_3\)-\(\text{CH}_2\text{OH}\), and after methylation the fatty acid composition was determined by gas-liquid chromatography (GLC). In each case percentage composition does not total to 100% because minor and unidentified fatty acids are not included. Unsaturation index is average no. of double bonds, and chain-length index is average no. of carbons in each fatty acid.

In other studies, either 10 mM PBN or 10 mM MNP was used as the spin trap. As opposed to results obtained with PBN in the same experiments, no radical adducts were detected when U937 cells enriched with DHA were exposed to 100 \(\mu\)M \(\text{FeSO}_4\) in the presence of either of these spin traps.

Ordinarily, the cells were washed with PBS before the initiation of radical formation. To exclude the possibility that the lipid radical might be formed from unesterified fatty acid that remained loosely bound to the cell surface, cells supplemented with 20 \(\mu\)M DHA were washed with PBS containing 10 \(\mu\)M bovine serum albumin before exposure to PBN and \(\text{FeSO}_4\). The radical adduct signal detected was the same height and shape as that obtained from corresponding DHA-enriched cells washed only with PBS (data not shown).

Cell viability was assessed by trypan blue dye exclusion after exposure to PBS containing \(\text{FeSO}_4\), PBN, \(\text{FeSO}_4\) plus PBN, or, as a control, PBS containing none of these additives. After these samples were placed in the EPR spectrometer and the spectral data were collected, the number of cells not stained by the dye was measured by microscopy. In each case, > 94% of the cells excluded the trypan blue, and no significant differences were observed between any of the treatments (data not shown).

**Effect of type of fatty acid supplement.** To determine whether polyunsaturated fatty acids other than DHA would also enhance the production of lipid radicals, the U937 cell growth media were supplemented with different fatty acids. Table 3 shows the fatty acid compositional differences that occurred in the total lipids extracted from the cells when the growth medium contained 20 \(\mu\)M of one of the following fatty acids: oleic acid \([18:1(n-9)]\), linoleic acid \([18:2(n-6)]\), linolenic acid \([18:3(n-6)]\), eicosatrienoic acid \([20:3(n-3)]\), arachidonic acid \([20:4(n-6)]\), eicosapentaenoic acid \([20:5(n-3)]\), or DHA \([22:6(n-3)]\). In each case supplementation with a fatty acid led to an increased proportion of that particular fatty acid in the cell lipids, but the degree of increase varied for individual fatty acids. Similar percentage

![Fig. 4](image-url)
increases occurred with linoleic acid, arachidonic acid, eicosatrienoic acid and DHA, and the composition of the cell lipids in each case was considerably different from the cells supplemented with oleic acid. Although less linolenic and eicosapentaenoic acids were present in the cell lipids after supplementation with these acids, substantial increases in their elongation products were detected. When the amounts of the elongated and unmodified fatty acids were added, the total enrichments obtained with both of these fatty acids was similar to those obtained with linoleic acid, arachidonic acid, eicosatrienoic acid, and DHA.

The percentage compositional changes reported in Table 3 reflect substantial changes in the amount of these fatty acids present in the cell lipids. For example, cells supplemented with arachidonic acid contained 123 ± 15 mg/20·4, 0.67 ± 0.3 mg/20·5, 60 ± 6 mg/22·4, and 6.3 ± 0.1 mg/22·5 per mg cell protein (n = 4). By contrast, those supplemented with eicosapentaenoic acid contained 14 + 2 mg/20·4, 92 + 11 mg/20·5, 3.3 + 0.8 mg/22·4, and 79 ± 15 mg/22·5 (n = 4). However, the total amounts of fatty acid present in the cell lipids were similar in both cases, 391 ± 33 compared with 399 ± 30 mg/mg cell protein, respectively. Cells enriched with oleic acid, which do not produce a clearly discernible radical adduct, also contained a similar amount of total fatty acid, 431 ± 13 mg/mg cell protein (n = 4).

When the various fatty acid-modified U937 cells were subjected to ferrous iron-mediated oxidant stress, radical adducts were detected by EPR spectroscopy (Fig. 5). The hyperfine splitting constants determined from the spectra produced by cells after supplementation with polyunsaturated fatty acids indicate that, as in the case of DHA supplementation, the radicals trapped by POBN are carbon-centered species. The adducts generated by the cells supplemented with eicosapentaenoic, arachidonic, and eicosatrienoic acids have hyperfine splitting values centered around $a_N = 15.66$ G and $a_H = 2.54$ G (Table 4). The low signal-to-background ratio precluded the extraction of accurate hyperfine splitting values from the spectra generated by cells supplemented with linolenic, linoleic, or oleic acids or the cells grown without fatty acid supplementation. However, the position and shape of these spectra resemble the spectra of the carbon-centered radical adducts generated by the DHA-enriched cells.

Spectra obtained with DHA-enriched cells contain enough information for successful computer simulation. A typical spectrum obtained from a suspension of DHA-enriched cells exposed to FeSO$_4$ in the presence of POBN is shown in Fig. 6A. The spectrum suggests that more than one species is present. The asymmetry in the width of the peaks precluded successful computer simulation. However, the simulation, Fig. 6B, provided the best fit when the model contained two radical species. Both simulated spectra are carbon-centered radical adducts of POBN, the first with splitting constants of $a_N = 15.66$ G and $a_H = 2.54$ G (Fig. 6C) and the second with $a_N = 15.94$ G and $a_H = 2.05$ G (Fig. 6D).
radical adducts generated during lipid peroxidation (5, 9, 10, 22).

The amounts of radical adduct trapped by POBN from cells enriched with DHA or eicosapentaenoic acid, calculated using the 3-carboxy proxyl standard, were greater than those obtained from cells enriched with the other fatty acids (Fig. 7). Comparisons with the fatty acids containing 20 carbons suggest that more radicals are trapped from cells enriched with the n-3 as opposed to n-6 fatty acids. Particularly important in this regard is the fact that more radicals were trapped when the cells were enriched with 20:3(n-3) than 20:4(n-6), even though the n-3 fatty acid contains one less double bond. However, as shown in Table 3, the lipids from the cells enriched with 20:3(n-3) contain slightly more average number of double bonds per fatty acyl chain than those from the cells enriched with 20:4(n-6), possibly accounting for the difference in spin adduct formation.

Location of trapped radical. A DHA-enriched cell suspension was treated with ferrous iron and the incubation medium was then rapidly separated from the cell pellet by centrifugation. The free radical spectrum detected in the intact system before centrifugation is shown in Fig. 8A. After the medium and cells were separated, however, the carbon-centered radical adduct was detected only in the supernatant solution and not in the cell pellet (Fig. 8, B and C). In a similar experiment, a DHA-enriched cell suspension was centrifuged initially to separate the cells from the medium, and each fraction was then exposed separately to ferrous iron-induced oxidant stress. No free radical was observed with the cell-free medium (Fig. 8D); a radical adduct was

Table 4. POBN spin adduct hyperfine splitting constants

<table>
<thead>
<tr>
<th>Fatty Acid Supplement</th>
<th>Hyperfine Splitting Constants, G</th>
<th>Experimental</th>
<th>Simulated radical 1</th>
<th>Simulated radical 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aN</td>
<td>aH</td>
<td>aN</td>
</tr>
<tr>
<td>Docosahexaenoic [22:6(n-3)]</td>
<td>15.61</td>
<td>2.74</td>
<td>15.85</td>
<td>2.61</td>
</tr>
<tr>
<td>Eicosapentaenoic [20:5(n-3)]</td>
<td>15.56</td>
<td>2.65</td>
<td>15.76</td>
<td>2.39</td>
</tr>
<tr>
<td>Arachidonic [20:4(n-6)]</td>
<td>15.52</td>
<td>2.58</td>
<td>15.79</td>
<td>2.35</td>
</tr>
<tr>
<td>Eicosatrienoic [20:3(n-3)]</td>
<td>15.52</td>
<td>2.62</td>
<td>15.91</td>
<td>2.18</td>
</tr>
</tbody>
</table>

Spectra were obtained from fatty acid-enriched cells by electron paramagnetic resonance spectrometry as described in METHODS. Computer simulations performed on these spectra indicated that composite spectra containing 2 radical adduct species produced excellent fits. Constants for column labeled experimental assume only 1 radical species. Values obtained represent average of 6 or more individual measurements made on each spectrum.
Fig. 6. Simulations of POBN spin adduct spectra. A: EPR spectrum of radical adducts detected at room temperature in solutions containing 2 x 10^6 U937 cells previously grown in a medium supplemented with 20 μM DHA, 10 mM POBN, and 100 μM FeSO₄ in a total volume of 0.5 ml air-saturated PBS. EPR spectrometer conditions were those listed under METHODS. B: computer-generated composite spectrum of spectra C, D, and E, which are simulations based on data obtained from spectrum A. C: simulated spectrum of carbon-centered POBN radical adduct: line width, 0.56 G; line shape, 53% Lorentzian-47% Gaussian; mole ratio, 0.58; α_N = 15.45 G, α_H = 2.89 G. D: simulated spectrum of carbon-centered POBN radical adduct: line width, 0.62 G; line shape, 70% Lorentzian-30% Gaussian; mole ratio, 0.36; α_N = 15.94 G, α_H = 2.05 G. E: simulated spectrum of POBN artifact: line width, 0.56 G; line shape, 25% Lorentzian-75% Gaussian; mole ratio, 0.06; α_N = 14.54 G, α_H = 14.44 G. This artifact arises from breakdown of POBN that can result in spin adduct species 2-methyl-2-nitrosopropane/H.

Fig. 7. Effect of fatty acid supplementation on amount of spin adduct produced. Fatty acid supplementation was done as described in Figure 5. Incubations at room temperature contained 2 x 10^6 fatty acid-enriched U937 cells, 10 mM POBN, and 100 μM FeSO₄ in a total volume of 0.5 ml PBS. Spin adduct amounts were determined by comparison with standard peak areas. Each bar represents results obtained from at least 3 separate experiments ± SE.

Effect of ascorbate. Ascorbate, which can serve as a pro-oxidant, was introduced into the system to increase the flux of free radicals produced by the FeSO₄-mediated oxidative stress (15). When only 20 μM FeSO₄ was added to a mixture containing 2 x 10^6 cells enriched with DHA, 25 mM POBN, and 100 μM ascorbate, an EPR spectrum was obtained exhibiting a composite of the lipid-derived radical adducts and the ascorbate radical (Fig. 9A). In the absence of ascorbate, this concentration of FeSO₄ is too low to produce an appreciable amount of lipid radicals under the conditions and instrument settings used (26). With ascorbate, the EPR signal intensity was increased substantially over that of spectra obtained with 100 μM FeSO₄ alone (Fig. 9B). However, as seen in Fig. 9A, the ascorbate radical
Fig. 8. Localization of POBN spin adducts in incubation mixture. Incubations were done at room temperature in 0.5 ml air-saturated PBS solutions containing 10 mM POBN and 2 × 10⁶ U937 cells that had been grown previously in medium supplemented with 20 μM DHA. A: intact cells and medium together after incubation with 100 μM FeSO₄. B: supernatant fraction of incubation described in A, after 5-min centrifugation at 15,000 g. C: pellet fraction of incubation described in A, after centrifugation. D: supernatant fraction, with 100 μM FeSO₄ added after medium was separated from cells. E: pellet fraction, with 100 μM FeSO₄ added to cells after they were separated from initial medium and resuspended.

A 100 μM Ascorbate + 20 μM FeSO₄

B 100 μM FeSO₄

Magnetic Flux Density

Fig. 9. Effect of presence of ascorbate on spectral intensity. U937 cells (2 × 10⁶) previously grown in medium supplemented with 20 μM DHA were placed in 0.5 ml air-saturated PBS containing 25 mM POBN. A: spectrum obtained after addition of 100 μM ascorbate and 20 μM FeSO₄. B: spectrum obtained in presence of 100 μM FeSO₄ without added ascorbate.

DISCUSSION

Lipid peroxidation is a complex free radical chain reaction initiated by the abstraction of a bis-allylic hydrogen atom from a polyunsaturated fatty acid (6). Initiation of this process requires the presence of iron or other catalytic metals (25, 34). The initiation mechanism itself is a complex process that probably involves hydrogen atom abstraction by either Fe²⁺-generated hydroxyl radicals (HO⁺), ferryl ions (FeO²⁺), perferryl molecules (Fe²⁺-O₂), or possibly an Fe²⁺-O₂-Fe³⁺ com-
The largest EPR signal intensity occurred when the cells were supplemented with eicosapentaenoic acid or DHA. In the case of DHA, POBN spin adducts were clearly visible when the supplemental fatty acid concentration to which the cells were exposed during growth was 5 μM. Under these conditions DHA comprised 11% of the U937 cell fatty acid content (Fig. 3). For comparison, the DHA content in the retina and brain usually exceeds 15% of the tissue fatty acids (2). Because radicals were clearly visible at DHA contents <15%, radical formation in the U937 cells almost certainly is not an artifact due to the intracellular accumulation of polyunsaturated fatty acid that is above physiological levels. Our interpretation is that relative increases in polyunsaturated fatty acid magnify the inherent process to a level high enough to be detected by the currently available EPR spin-trapping techniques. The increase in spin adduct formation was roughly parallel to the increase in cellular DHA content (cf. Figs. 2 and 3). This is consistent with the interpretation that the extent of radical formation in response to a given level of oxidant stress depends on the availability of polyunsaturated substrate in the cell lipids. Studies with [1-14C]DHA indicate that the U937 cells channel the newly incorporated fatty acid into three lipids, phosphatidylcholine, phosphatidylethanolamine and triglycerides. It is not therefore possible to distinguish which intracellular location to trap the radicals that were produced.

When oxidant stress was initiated with FeSO₄ alone, the signal-to-background ratio of the POBN spin adducts was very low. The radical adduct signals were enhanced substantially by adding ascorbate to increase the flux of radical generation. Ascorbate usually serves as a biological antioxidant (30), but it also can serve as a prooxidant (15). The ascorbate monoanion (AscH⁻), the oxidizable and thus can serve as a reducing agent (6, 7) for ethane collection. The quantitative relationship between the two processes is difficult to assess, however, because radical formation was measured within 5 min, whereas 2 h were required for ethane collection.

Simulations of the EPR spectra were undertaken in an attempt to begin characterizing the lipid radicals formed by the intact cells. These simulations provide information about the minimum number of radical species that constitute the spectrum, their relative amounts and the hyperfine coupling constants. The simulations indicate that more than one carbon-centered radical was trapped when the U937 cells were enriched with either the n-3 or n-6 polyunsaturated fatty acids. On the basis of chemical studies reported by others, we propose that the radicals trapped by POBN are formed by P-scission of the alkoxyl radical, LO₂⁺, which is oxidized while reducing Fe³⁺ to Fe²⁺. The resulting Fe²⁺ can initiate another round of free radical oxidation reactions by generating one electron oxidants such as HO·, ferryl ions, perfferryl species, or an Fe²⁺•O₂•Fe³⁺ complex under these experimental conditions and thereby increase lipid radical formation.

The fact that spin adducts were trapped in the extracellular fluid suggests that the actions of these lipid-derived carbon-centered radicals may not be confined just to the cells exposed to the oxidant stress. In
this regard, other free radicals also are released by cells, including superoxide (31) and nitric oxide (11), and they have effects in the surrounding medium or adjacent cells (3, 19, 32). The homogenate experiments indicate that the failure to detect spin adducts in the intact cell pellet is not due to their destruction or quenching of the signal by the intracellular contents. The vital dye studies indicate that exposure to POBN and iron was not acutely cytotoxic, making it unlikely that the radicals or spin adducts were released into the medium due to cell damage. Also, the assay done on isolated medium after it had been incubated with the cells (Fig. 8D) demonstrates that the radicals are not generated in the medium from lipids or particles released from the fatty acid-enriched cells. If the radicals are trapped intracellularly and the spin adducts quickly extruded from the cells, the detection of the adducts in the medium would not mean that the radicals themselves could be released from the cells. An equally plausible explanation of the present results, however, is that some of the lipid radicals are either released into the extracellular fluid and trapped there or generated in membranes that are exposed to the extracellular fluid and trapped at the cell surface. Because of the very short life of carbon-centered radicals, the possibility that they are generated near the cell surface seems more likely. This is consistent with the finding that the microsome fraction, which contains plasma membranes when isolated by the procedure utilized (33), is highly enriched in polyunsaturated fatty acids if the cells were grown in the presence of supplemental DHA (Table 2). Also, some POBN would be expected to associate with the surface membrane, since it is an amphipathic substance. If so, it would be properly located to trap any radicals generated in the plasma membrane. Based on these considerations, the present observations suggest that at least some lipid radicals formed in response to oxidant stress may be generated in a location where they are able to exert effects on extracellular structures. Such a process might have functional relevance even if it represents only a small fraction of the total lipid radicals produced.

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