Polyunsaturated fatty acids increase lipid radical formation induced by oxidant stress in endothelial cells

Lynett S. Alexander-North,* James A. North,* Kitonga P. Kiminyo,* Garry R. Buettner,† and Arthur A. Spector[1,*]

Department of Biochemistry* and Electron Spin Resonance Facility,† College of Medicine, University of Iowa, Iowa City, IA 52242

Abstract Lipid-derived free radicals were detected by electron paramagnetic resonance (EPR) spectrometry when cultured endothelial cells attached to Cytodex beads were exposed to iron-induced oxidant stress in the presence of the spin trap α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (POBN). Radical adduct formation was enhanced greatly when the cells were supplemented during growth with polyunsaturated fatty acids. The largest EPR signal intensity was observed in cells enriched with docosahexaenoic acid (DHA) or eicosapentaenoic acid, but enhanced radical adduct production also occurred after exposure to arachidonic, α-linolenic, γ-linolenic, or linoleic acids. Radical adduct formation increased as the DHA content of the cells increased and approached a maximum after only 6 h of exposure to DHA. Ascorbic acid, acting as a pro-oxidant, enhanced radical adduct formation in cells enriched with DHA. The EPR signal intensity was reduced when the cells were tested 6 h after replacement of the DHA-enriched medium with a medium containing 5–20 μM oleic acid, indicating that the increased endothelial responsiveness to oxidant stress is reversible. Likewise, when U937 monocytes enriched with DHA were exposed subsequently to 20 μM oleic acid, a 35–45% decrease in radical adduct formation also occurred. These findings suggest that the endothelium may become more susceptible to oxidative injury when it is exposed to elevated amounts of polyunsaturated fatty acids. However, the effect appears to be temporary. The protective action of oleic acid against oxidant stress is not confined to the endothelium; it applies to monocytes as well.—**Alexander-North, L. S., J. A. North, K. P. Kiminyo, G. R. Buettner, and A. A. Spector.** Polyunsaturated fatty acids increase lipid radical formation induced by oxidant stress in endothelial cells. *J. Lipid Res.* 1994. **35:** 1773–1785.

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Free radicals can cause oxidative damage to the vascular endothelium (1). The radicals can be generated by the endothelial cells themselves (2, 3), or they can be released by leukocytes that come in contact with the vascular wall (4). Exposure to free radicals can produce endothelial dysfunction, leading to excessive vascular permeability (5), abnormalities in the fibrinolytic system (6), and decreases in prostacyclin production (7, 8). Most studies have focused on reactive oxygen-species such as hydrogen peroxide (6, 7) or superoxide (2, 9), which probably produce endothelial damage by generating hydroxyl radicals that are formed from these oxidants in reactions catalyzed by iron (9–11).

Recent work with U937 monocytes, a cultured cell line derived from a human monocytic leukemia (12), has demonstrated the formation of carbon-centered free radicals during lipid peroxidation induced by iron (13). These radicals were detected by electron paramagnetic resonance (EPR) spectrometry when a suspension of intact cells was incubated with ferrous ions (Fe^{2+}) in the presence of a spin trap, α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (POBN). Spin adduct formation was enhanced greatly when the U937 cells were enriched during growth with docosahexaenoic acid (DHA, 22:6),[2] an omega-3 polyunsaturated fatty acid containing six double bonds. By contrast, radical adduct formation was not increased above the background level when the cells were grown in medium supplemented with oleic acid (18:1). The characteristics of the EPR spectrum indicate that the spin adducts contain a short-chain alkyl radical, probably an ethyl, pentyl or pentenyl group (14, 15), that is derived from the cleavage of the alkoxyl radical formed from

Abbreviations: EPR, electron paramagnetic resonance; Fe^{2+}, ferrous ions; POBN, α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone; DHA or 22:6, docosahexaenoic acid; DMEM, Dulbecco's minimal essential medium; GLC, gas-liquid chromatography; 3-CP, 3-carboxy proxyl; PPAEC, porcine pulmonary artery endothelial cells; BAEC, bovine aortic endothelial cells; FBS, fetal bovine serum; TBARS, thiobarbituric acid reactive substances; HPLC, high performance liquid chromatography; EPA or 20:5, eicosapentaenoic acid.

[1]To whom correspondence should be addressed.

[2]The structure of each fatty acid is indicated as number of carbon atoms:number of double bonds.

peroxidized fatty acyl chains (13). Because of the apparent role of free radicals in endothelial injury and certain vascular diseases (1–9), we wished to determine whether a similar carbon-centered radical is generated when endothelial cells are exposed to oxidant stress. Such a response could not be predicted to occur with certainty in endothelial cells as the process might be dependent on the fact that the U937 cells are monocytes and are neoplastic.

To make spin trapping measurements by the presently available procedures, the cells must be placed in the EPR spectrometer as a suspension. Because endothelial cells normally grow as an adherent culture, they would have to be mechanically or chemically detached to form a suitable suspension. Such disruption might cause cell damage and thereby lead to free radical production. To avoid this potential difficulty, an approach was developed recently for applying the EPR procedure to cells such as the endothelium that are normally attached to surfaces. This involves growing the cells on microcarrier beads and adding the bead suspension directly to the EPR aqueous flat cell, without disrupting the surface attachment and polarization of the cells (9). Using this approach, we have detected POBN radical adducts when intact endothelial cells were exposed to Fe^{2+}-induced oxidant stress. The intensity of radical adduct formation increased considerably when the cultures were supplemented during growth with polyunsaturated fatty acids. These findings suggest that exposure to high levels of polyunsaturated fatty acid can increase the response of endothelial cells to oxidant stress and thereby make the vascular wall more susceptible to free radical-induced damage.

METHODS

Materials

Iminodiacetic acid chelating resin, Cytodex-3 microcarrier beads, POBN, and all other spin traps were obtained from Sigma Chemical Co. (St. Louis, MO). Medium M199 and Dulbecco's Minimal Essential Medium (DMEM) were purchased from Gibco BRL (Grand Island, NY). Methyl heptadecanoate and SP2330 gas–liquid chromatography (GLC) column packing material were supplied by Supelco (Bellefonte, PA), 3-carboxy proxyl (3-CP) by Aldrich (Milwaukee, WI), and fatty acids by Nu-Chek Prep (Elysian, MN). All reagents were HPLC grade.

Cell culture and incubations

Porcine pulmonary artery endothelial cells (PPAEC), which were studied in most of the experiments, and bovine aortic endothelial cells (BAEC) were obtained as described previously (9, 16). Stock cultures were maintained in modified M199 medium containing 2 mM L-glutamine, 100 μM nonessential amino acid mixture, 10 μl/ml basal medium Eagle vitamins, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin sulfate. The PPAEC medium was supplemented with 10% fetal bovine serum (FBS); the BAEC medium with 20% FBS. Spinner cultures were prepared by the procedure of Britigan, Roeder, and Shasby (9). The cells were detached by exposure of the confluent stock cultures to a solution containing 0.25% trypsin and 0.1% ethylenediamine tetraacetate. After sedimenting the cells and washing with trypsin-free medium, the cells were mixed with 130–135 μM Cytodex-3 microcarrier beads coated with porcine skin collagen. The beads had been hydrated for 3 h at 37°C, autoclaved for 20 min, and washed with medium containing 10% FBS before being added to the spinner flasks. Approximately 6–7 × 10^6 cells were added per 100 mg beads. The mixture was rotated mechanically for 3 min and allowed to settle for 20 min. These cycles were repeated for 2 h. The medium was then brought to a final volume of 100–120 ml, and spinning was maintained at 40–45 rpm in a 37°C incubator with a gas phase of 5% CO_2 in air.

The endothelial cells reached near confluence on the beads in 3 days. Supplemental fatty acid in ethanol was added to DMEM containing 2.5% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin sulfate. The maximum ethanol concentration was 0.3% (v/v); a corresponding amount of ethanol was added to the unsupplemented medium of the control cultures. After 24 h of incubation with stirring, the beads were allowed to settle and then were aspirated by pipet, sedimented, and washed 5 times with chelating resin-treated phosphate buffer. This stock solution, which contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4, and 1.5 mM KH_2PO_4, was stirred overnight with chelating resin to remove reactive trace metals (17). The absence of catalytic metals was verified by testing with ascorbate (18).

After the final wash, the cell-covered beads were suspended in an amount of chelating resin-treated phosphate buffer equal to the packed bead volume. A 0.5 ml aliquot of the washed beads was placed in a 1.5 ml microcentrifuge tube and, in most of the experiments, 25 μl of 200 mM POBN was added so that the concentration of the spin trap in the final incubation mixture would be 10 mM. Unless indicated otherwise, 100 μM $FeSO_4$ was added to initiate lipid peroxidation (13). This stock solution consisted of 10 mM $FeSO_4 \cdot H_2O$ in 10 ml H_2O containing 50 μl concentrated H_2SO_4, added to stabilize the iron as Fe^{2+}. In the one set of experiments in which 200 μM ascorbate also was added to the incubation mixture, $FeCl_3$ was used instead of $FeSO_4$, and the final concentrations of POBN and $FeCl_3$ were 25 and 20 μM, respectively. The order of addition was cell-covered beads, POBN, and ascorbate where indicated. The iron was always added last, immediately before the start of the EPR measurements.

The human U937 monocytic leukemia cell line was used for one series of experiments. These cells were grown as previously described in a humidified atmosphere of 95% air: 5% CO_2 at 37°C in a medium containing RPMI 1640 supplemented with 5% FBS, 15 mM HEPES, and 2 mM L-glutamine (13). The cells were maintained as an exponentially growing culture by passage every 2–3 days. As the U937 cells grow as a suspension, they were added directly to the EPR flat cell without the use of microcarrier beads.

EPR measurements

EPR spectra were obtained with a Bruker ESP 300 spectrometer operating at 9.79 GHz and 100 kHz modulation frequency (13). Air-saturated samples were placed in a quartz flat cell centered inside a TM_{110} cavity at room temperature. From 1 to 2 min elapsed between the addition of the iron and the start of the EPR measurement, and the spectra were recorded over the next 335 sec. The spectrometer settings were: microwave power, 40 milliwatts (19); modulation amplitude, 1.05 G; time constant, 1.3 s; scan rate, 60 G/335 s; receiver gain, 1×10^6. Quantitative values were calculated from the EPR spectra using the stable free radical, 3-CP, as the standard. Based on the correlation between several 3-CP concentrations and the resulting peak ranges of the low field signal, we calculated a coefficient relating peak height to quantity of POBN radical adduct.

Chemical separations and assays

Protein content was measured by a modification of the Lowry method in which sodium dodecylsulfate was added to solubilize lipids (20). A final centrifugation step was also needed to sediment the beads, in order to prevent them from entering the light path of the spectrophotometer. Color development was terminated after 25 min, and the beads were sedimented at room temperature for 5 min at 2000 *g* prior to measurement of absorbance at 750 nm.

The fatty acid composition of the cells was determined by GLC. An aliquot of the washed cell-bead suspension was extracted with a chloroform–methanol mixture (21). Polar and neutral lipids were separated from this extract by chromatography on 10 × 10 mm silicic acid columns (22). The columns were prepared by washing with n-heptane. The lipid samples were loaded onto the column in 0.15 ml diethyl ether–heptane 1:3 (v/v), and the column was washed with 1 ml heptane. Neutral lipids were eluted with 6 ml chloroform–methanol 100:2, and polar lipids with 8 ml methanol–water 100:2.

After drying under a stream of N_2, lipid samples were saponified in 33% KOH–95% ethanol 1:20 at 70°C for 45 min. The samples were extracted with heptane, acidified, extracted 3 times with 1 ml heptane to isolate the fatty acids, and the three extracts were combined. The dried residue was methylated for 10 min at 95°C with 10% BF_3 in methanol (23), and the mixture was extracted 3 times with 1 ml heptane. After the heptane extracts were combined and dried, the fatty acid methyl esters were dissolved in CS_2.

GLC separation was obtained with a Hewlett-Packard 5890 system containing a 1.9 m × 2 mm glass column packed with SP2330 on 100–120 mesh Gaschrome WAW (13). The heated on-column injector and flame ionization detector were maintained at 250°C, and the oven was programmed to increase from 165 to 220°C. The data were integrated with a Hewlett-Packard 3993A integrator and analyzed by computer to determine the weight percentage composition of the fatty acid methyl ester mixture. A measured quantity of heptadecanoic acid (17:0) was added as an internal standard so that the quantity of fatty acids contained in each sample also could be calculated.

Thiobarbituric acid reactive substances (TBARS) were measured in the endothelial cells by a fluorescence assay (24). The reagent solution contained 0.5 g thiobarbituric acid and 0.3 g sodium dodecylsulfate dissolved in 100 ml H_2O. After 1.5 ml of this solution was incubated with cells suspended in 1.6 ml 0.2 M glycine• HCl containing 100 μM butylated hydroxytoluene for 15 min at 100°C, the mixture was cooled and 1 ml glacial acetic acid and 2 ml chloroform were added (25). The aqueous phase was removed, passed through a 0.22 μ filter, and the fluorescence was measured at excitation and emission wavelengths of 532 and 555 nm, respectively. The assay was standardized with 1,1,3,3-tetramethoxypropane.

Radioactive fatty acid methyl esters and related metabolic products were separated by a high performance liquid chromatography (HPLC) gradient procedure using a 150 × 2.1 mm i.d. Absorbosphere C-18 reverse phase column (Alltech Associates, Inc., Deerfield, IL). After incubation with [1-^{14}C]DHA, the cells and the medium were separated, and the lipids were extracted and methylated with 12% BF_3 in methanol (23). The methyl esters were extracted, dried under N_2, dissolved in acetonitrile, and injected in this solvent. Elution was initiated with a mixture of 60% acetonitrile and H_2O containing H_3PO_4, pH 3.5. After 1.5 min, the acetonitrile was increased linearly to 85% over 14.5 min, then to 100% over the next 8 min. The elution continued at 100% acetonitrile to 50 min. Under these conditions, the retention time of [1-^{14}C]DHA varied from 23.8 to 24.8 min in various chromatograms. Radioactivity was measured by mixing the column effluent with BudgetSolve scintillator solution (Research Products International Corp., Mount Prospect, IL) and passing the mixture through an on-line Radiomatic Flo-one Beta detector.

RESULTS

Detection of lipid radicals

Our EPR spin trapping studies demonstrated the formation of radical adducts when endothelial cells adherent to microcarrier beads were subjected to oxidant stress induced by exposure to 100 μM Fe^{2+}. **Figure 1** shows EPR spectra obtained with PPAEC and the spin trap POBN. A radical adduct was clearly visible when the PPAEC were enriched during growth with DHA (Fig. 1A). In these and all other studies, the growth medium was removed and the cell-covered beads were washed and placed in chelating resin-treated phosphate buffer prior to the EPR assay. Thus, the cells were not exposed to supplemental fatty acid, serum, or other nutrients during the time that oxidant stress was induced and the EPR signal was recorded. The POBN spin adduct had hyperfine splitting constants of a^N = 15.6 and a^H = 2.7. On the basis of previous studies in cell-free systems (14, 15), this spectrum has been assigned to alkyl radical adducts, probably ethyl, pentyl, or pentenyl radicals. No spin adducts were detected if PPAEC enriched with DHA were not exposed to Fe^{2+} (Fig. 1B). At the instrument settings used, the signals produced in response to Fe^{2+} when PPAEC were enriched with oleic acid (Fig. 1C) or grown without fatty acid supplementation (Fig. 1E) were not clearly distinguishable from the corresponding background spectra obtained when the cells were not exposed to Fe^{2+} (Fig. 1D and F).

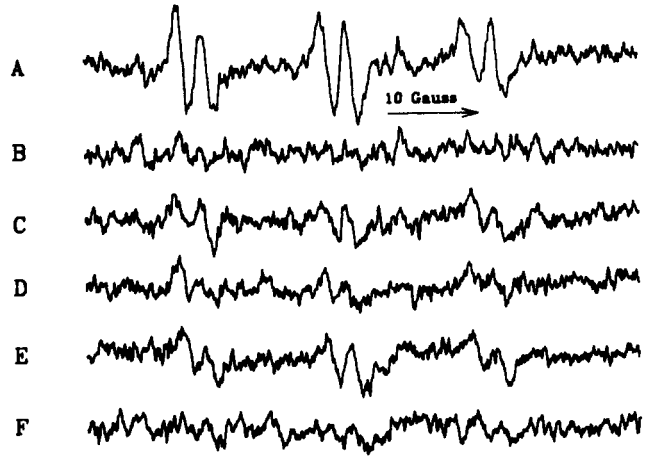

Fig. 1. EPR spectra of POBN lipid radical adducts produced by PPAEC. Endothelial cells grown to near confluence on Cytodex-3 microcarrier beads were incubated for 24 h in media containing either 15 μM DHA or oleic acid as a supplement, or no supplemental fatty acid. The beads were isolated, washed, and incubated at room temperature with 10 mM POBN and 100 μM $FeSO_4$ in a total volume of 0.5 ml chelating resin-treated phosphate buffer to obtain the EPR spectra. A: complete system with DHA-supplemented cells; B: DHA-supplemented cells without $FeSO_4$; C: complete system with oleic acid-supplemented cells; D: oleic acid-supplemented cells without $FeSO_4$; E: complete system with cells grown in medium containing no fatty acid supplement; F: unsupplemented cells without $FeSO_4$.

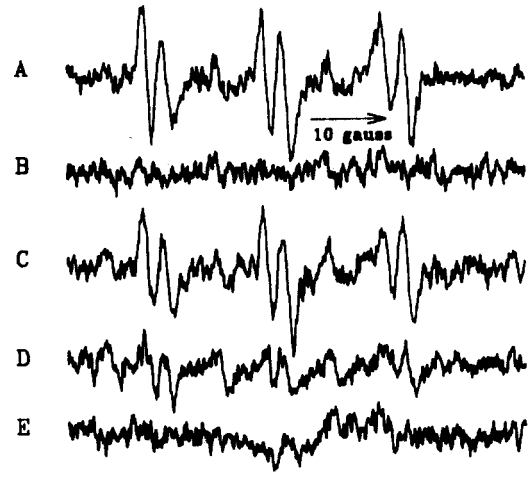

Fig. 2. EPR spectra of POBN lipid radical adducts produced by BAEC. The conditions were the same as described in Fig. 1. A: complete system with DHA-supplemented cells; B: DHA-supplemented cells without $FeSO_4$; C: complete system with EPA-supplemented cells; D: EPA-supplemented cells without $FeSO_4$; E: complete system with cells grown in a medium containing no fatty acid supplement.

Radical adducts also were detected in incubations with BAEC attached to microcarrier beads. As shown in **Fig. 2,** a carbon-centered POBN spin adduct was observed when BAEC enriched with either 15 μM DHA (Fig. 2A) or eicosapentaenoic acid (EPA) (Fig. 2C) were exposed to Fe^{2+}. These fatty acid-enriched cells did not produce detectable spin adducts in the absence of Fe^{2+} (Figs. 2B and D). Likewise, spin adducts were not clearly discernible when BAEC grown in an unsupplemented medium were exposed to Fe^{2+} (Fig. 2E).

In an attempt to obtain additional information about the type of radicals produced, spin traps other than POBN also were tested. BAEC supplemented with DHA were incubated with 25 mM spin trap and 100 μM $FeSO_4$ using the procedure described for POBN. The spin traps tested were N-t-butyl-α-phenylnitrone, 2-methyl-2-nitrosopropane, 3,5-dibromo-4-nitrosobenzenesulfonic acid, and nitrosodisulfonic acid. Corresponding incubations with POBN were included in these studies. A distinct radical adduct spectrum similar to that seen in Fig. 2A was observed when POBN was present, but no radical adducts were detected with any of the other spin traps.

A more intense EPR radical adduct signal was obtained from PPAEC enriched with DHA when radical formation was measured in a medium containing 20 μM $FeCl_3$ and ascorbate (**Fig. 3A**). No signal was obtained when $FeCl_3$ and ascorbate were omitted from the incubation mixture (Fig. 3B), or when $FeCl_3$ was added but ascorbate was omitted (Fig. 3D). Only a very weak signal was produced by PPAEC that did not receive supplemental fatty acid (Fig. 3E). As shown in Fig. 3C, the ascorbate radical (a^H = 1.8 G) produced a signal that overlapped the midfield doublet of the lipid-derived spin adduct.

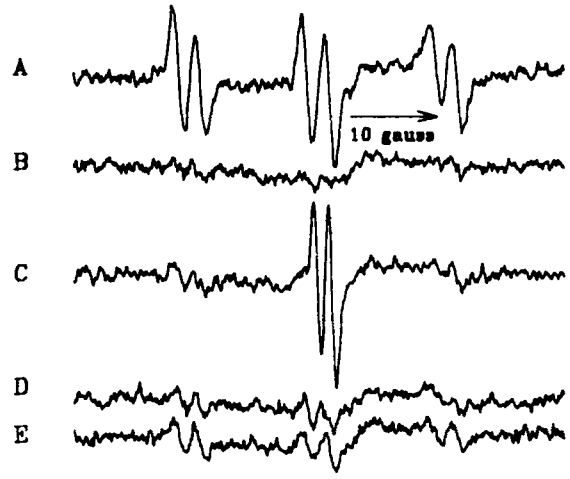

Fig. 3. Effect of ascorbate on radical adduct formation. PPAEC were prepared as described in Fig. 1. The EPR incubation mixture contained 10 mM POBN, 200 μM ascorbate, and 20 μM $FeCl_3$ in a total volume of 0.5 ml chelating resin-treated phosphate buffer. A: complete system with DHA-supplemented cells; B: DHA-supplemented cells in buffer alone; C: DHA-supplemented cells plus ascorbate, no $FeCl_3$; D: DHA-supplemented cells plus $FeCl_3$, no ascorbate; E: complete system with cells grown in a medium containing no fatty acid supplement.

Similar results were obtained when ascorbate was added to incubations with DHA-enriched BAEC. Because the presence of ascorbate might interfere with interpretations of the lipid radical spectra, ascorbate was not used in subsequent experiments.

DHA incorporation

Ultraviolet absorption spectra of the DHA added to the cell cultures were obtained to determine whether these preparations contained oxidized products. No absorbance maxima were detected between 225 and 300 nm in solutions containing 1.3–16.6 mM DHA, indicating that the DHA preparations did not contain measurable amounts of conjugated dienes. Likewise, no radioactive metabolites were detected by HPLC when [1-^{14}C]DHA was incubated for up to 24 h in a culture without cells (data not shown), indicating that no significant oxidation products were generated by contact with components contained in the growth medium.

To test the possibility that the putative oxidation products might be formed during exposure of DHA to the cells, BAEC and PPAEC were incubated with [1-^{14}C]DHA for up to 24 h. At various intervals, the cells and medium were separated, extracted, and the fatty acids contained in the medium and cell lipids were transesterified with BF_3 in methanol (23). The resulting fatty acid methyl esters were separated by HPLC, and radioactivity was detected with a flow scintillation counter. **Figure 4** contains representative chromatograms from this study. Fig. 4A illustrates that when the medium was added to the cultures, a single radioactive component with a retention time of 24.8 min, the same as that of a DHA methyl ester standard, was the only radioactive material detected. A similar result was obtained when the medium was assayed after 1 h of incubation with PPAEC (Fig. 4B). Although much less labeled material was present after 5 h of incubation, the radioactivity remaining in the medium still eluted as a single peak with the retention time of the DHA methyl ester (Fig. 4C). No polar radioactive metabolites were detected in the medium when the incubation was extended to 24 h. Similar results indicating the absence of radioactive DHA oxidation products in the medium were obtained in corresponding incubations with BAEC (data not shown).

The radioactive fatty acids present in the cell lipids during incubation with [1-^{14}C]DHA were also assayed by HPLC. In PPAEC, unmodified DHA accounted for 84%

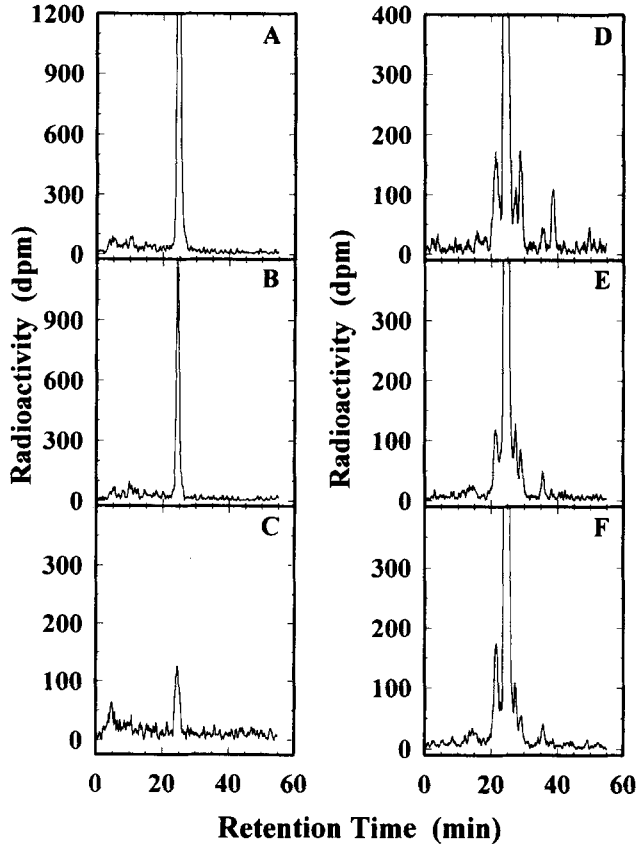

Fig. 4. Metabolic products formed during incubation of endothelial cells with DHA. Endothelial cultures were incubated for 1–24 h with a growth medium containing 20 μM [1-^{14}C]DHA. At various times, the medium and the cells were separated, and their lipids were extracted, methylated, and assayed for radioactivity by reverse phase HPLC with an on-line flow scintillation counter. The experiment was repeated twice with PPAEC and BAEC, and representative chromatograms of the radioactive products contained in extracts from one of the experiments are shown in this figure. A: Medium that was initially added to the cells; B: medium after incubation with PPAEC for 1 h; C: medium after incubation with PPAEC for 5 h; D: PPAEC after 24 h incubation; E: BAEC after 5 h incubation; F: BAEC after 24 h incubation. Note that the scale of the Y-axis in A and B is different from that in C–F.

TABLE 1. Fatty acid distribution of PPAEC grown on microcarrier beads

Fatty Acid[a]	Amount of Fatty Acid[b]			
	Unsupplemented	DHA Supplemented[c]	Unsupplemented	DHA Supplemented[c]
	% of total fatty acids		*μg fatty acid/mg protein*	
14:0	1.5 ± 0.5	2.0 ± 0.05	1.8 ± 0.3	3.6 ± 0.5[d]
16:0	20.4 ± 0.1	21.8 ± 0.5[d]	25.6 ± 4.1	38.6 ± 5.8
16:1	6.9 ± 0.5	4.7 ± 0.4[d]	9.0 ± 1.5	8.3 ± 1.4
18:0	15.3 ± 0.1	13.8 ± 0.7	19.3 ± 3.1	21.2 ± 3.0
18:1	31.0 ± 0.7	21.4 ± 0.7[d]	39.0 ± 6.3	39.5 ± 6.7
18:2	1.7 ± 0.1	1.3 ± 0.1[d]	2.1 ± 0.4	2.4 ± 0.5
18:3	1.3 ± 0.1	1.0 ± 0.1	1.6 ± 0.3	1.8 ± 0.3
20:3	1.2 ± 0.1	1.1 ± 0.2	1.5 ± 0.3	2.6 ± 0.7
20:4	6.3 ± 0.3	5.1 ± 0.2[d]	8.0 ± 1.3	9.6 ± 1.8
20:5	0.7 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	1.1 ± 0.3
22:4	2.2 ± 0.3	2.0 ± 0.3	2.6 ± 0.5	3.8 ± 1.0
22:5	1.9 ± 0.2	1.6 ± 0.04	2.3 ± 0.4	3.0 ± 0.5
22:6	3.1 ± 0.3	14.2 ± 0.6[d]	3.9 ± 0.7	25.8 ± 4.1[d]
Other	6.9	9.4		

[a] Fatty acids are designated by the number of carbons: number of double bonds.
[b] Results are expressed as the mean ± standard error of values obtained from four separate experiments for the unsupplemented cells and six separate experiments for the cells supplemented with DHA. The percentage values do not total to 100% because fatty acids accounting for < 0.5% and unidentified peaks are not included.
[c] Cells grown for 2 days in medium containing 15 μM DHA as a supplement.
[d] Significantly different from unsupplemented cells at $P < 0.05$.

of the cell-associated radioactivity at the end of the 24 h incubation, but some additional labeled peaks also were detected (Fig. 4D). These metabolites were not identified. However, the component with a retention time of 29.9 min, which comprised 3.1% of the radioactivity, co-chromatographed with a 24:6n-3 methyl ester standard.[3] Thus, some of the less polar labeled metabolites may be DHA elongation products. However, 4.3% of the radioactivity eluted with a retention time of 22.7 min. As the DHA was labeled in the carboxyl carbon, it is possible that this more polar compound may be a DHA oxidation product.

Similar results were obtained with the BAEC. After 5 h of incubation (Fig. 4E), 91% of the radioactivity remained as DHA, 2.7% had the same retention time as the 24:6 methyl ester standard, and 4% eluted ahead of DHA. The radioactivity contained in this more polar peak increased to 5.6% after incubation for 24 h (Fig. 4F). Therefore, while most of the radioactivity incorporated into the BAEC and PPAEC lipids remained as DHA, some metabolic products accumulated, and one of them had the HPLC elution properties consistent with a DHA oxidation product.

Supplementation of the growth medium with DHA produced changes in the fatty acid composition of the PPAEC. The data obtained by GLC are shown in **Table 1.** The main differences resulting from DHA supplementation were a 4-fold increase in the percentage of 22:6 and a decrease in 18:1. Based on values obtained with the internal standard, the quantity of each fatty acid present in the sample was also calculated. The unsupplemented cells contained 117 μg total fatty acid/mg protein, whereas those supplemented with DHA contained 161 μg/mg cell protein. This increase is due to the accumulation of triglyceride, which is synthesized intracellularly, as a storage form of excess fatty acid when endothelial cultures are exposed to fatty acid supplements (26). There was a large increase in 22:6 content in the cells supplemented with DHA, and the amount of total polyunsaturated fatty acid increased from 23 to 54 μg/mg protein. However, the absolute quantities of 16:1 and 18:1 were not reduced, only their relative abundances were decreased.

Changes in fatty acid composition did not occur only because triglyceride accumulated in the endothelial cells. As shown in **Table 2,** the fatty acid composition of the phospholipids was also modified in the PPAEC supplemented with DHA. The major changes were a 5-fold increase in the percentage of 22:6, and a 25% decrease in the percentage of 18:1.

Effect of DHA supplementation conditions

Only a relatively short incubation in medium containing supplemental DHA was needed to enhance POBN radical adduct formation in the PPAEC. **Figure 5 (top)** illustrates the time-dependence of the process. PPAEC attached to microcarrier beads were incubated for 1–24 h with medium containing 20 μM DHA supplement. After removal of this medium and resuspension of the PPAEC-

[3] Synthesized and provided by Dr. Howard Sprecher.

TABLE 2. Fatty acid distribution in phospholipids of PPAEC grown on beads

Fatty Acid	Fatty Acid Composition of Phospholipids[a]	
	Unsupplemented	DHA Supplemented[b]
	% of total fatty acids	
14:0	0.9 ± 0.1	1.5 ± 0.2
16:0	18.0 ± 0.4	20.2 ± 0.1[c]
16:1	7.4 ± 0.2	5.5 ± 0.6[c]
18:0	17.3 ± 1.7	15.7 ± 0.6
18:1	32.1 ± 1.3	23.8 ± 0.7[c]
18:2	1.4 ± 0.1	1.1 ± 0.1
18:3	0.2 ± 0.1	0.1 ± 0.1
20:1	1.2 ± 0.1	0.8 ± 0.0[c]
20:4	6.4 ± 0.6	5.6 ± 0.3
20:5	0.2 ± 0.1	0.9 ± 0.0[c]
22:4	2.0 ± 0.3	1.5 ± 0.2
22:5	1.9 ± 0.3	1.7 ± 0.2
22:6	3.2 ± 0.4	15.6 ± 0.9[c]
Rest	8.0	5.9

[a] Results are expressed as the mean ± standard error of values obtained from three separate experiments. The values do not total to 100% because fatty acids that account for < 0.5% and unidentified peaks are not included.

[b] Cells grown for 2 days in a medium containing 15 μM DHA as a supplement.

[c] Significantly different from unsupplemented cells at $P < 0.05$.

covered beads in chelating resin-treated phosphate buffer, oxidant stress was induced with Fe^{2+}, and POBN spin adduct formation was measured. An increase in spin adducts was observed when the cells were exposed to supplemental DHA for only 1 h, and the maximum value was approached within 6 h of incubation.

These spin adduct data are consistent with the time-dependent changes that were observed in PPAEC fatty acid composition. As seen in Fig. 5 (bottom), the 22:6 and total polyunsaturated fatty acid percentages reached maximum values within 6 h of incubation in medium supplemented with 20 μM DHA. The percentage of 18:1 also decreased somewhat, but the largest decrease in 18:1 occurred between 6 and 24 h.

Radical adduct formation also was dependent on the concentration of DHA added to the growth medium. **Figure 6 (top)** shows that only a relatively small increase occurred in Fe^{2+}-induced spin adduct formation when the DHA concentration of the growth medium was raised from 0 to 15 μM. A sharp increase occurred, however, when the concentration was increased from 15 to 20 μM. Fig. 6 (bottom) illustrates that, by contrast, the percentage of 22:6 and total polyunsaturated fatty acids in the PPAEC lipids increased roughly linearly over this range of DHA concentrations. Likewise, the percentage of 18:1 decreased linearly. Therefore, as opposed to radical formation, no abrupt changes in fatty acid composition occurred when the DHA concentration of the growth medium exceeded 15 μM. Above 20 μM, the PPAEC began to detach from the microcarrier beads, so that only a limited range of DHA concentrations could be explored in these studies.

Lipid peroxidation

TBARS were detected in the DHA-enriched cells grown in 9.6 cm^2 tissue culture wells following a 1-h incubation with 100 μM $FeSO_4$. A TBARS value of 92 ± 6.4 nmol (n = 3) was obtained with the PPAEC cultures; this was reduced to 79 ± 3.3 when 10 mM POBN was present in the medium during the 1-h incubation. In a similar study with BAEC, the values obtained after a 1-h incubation with the $FeSO_4$ were 74 ± 11 nmol (n = 3) in the absence of any spin trap, and 51 ± 9.3 when POBN was present. In a second experiment, the TBARS values were

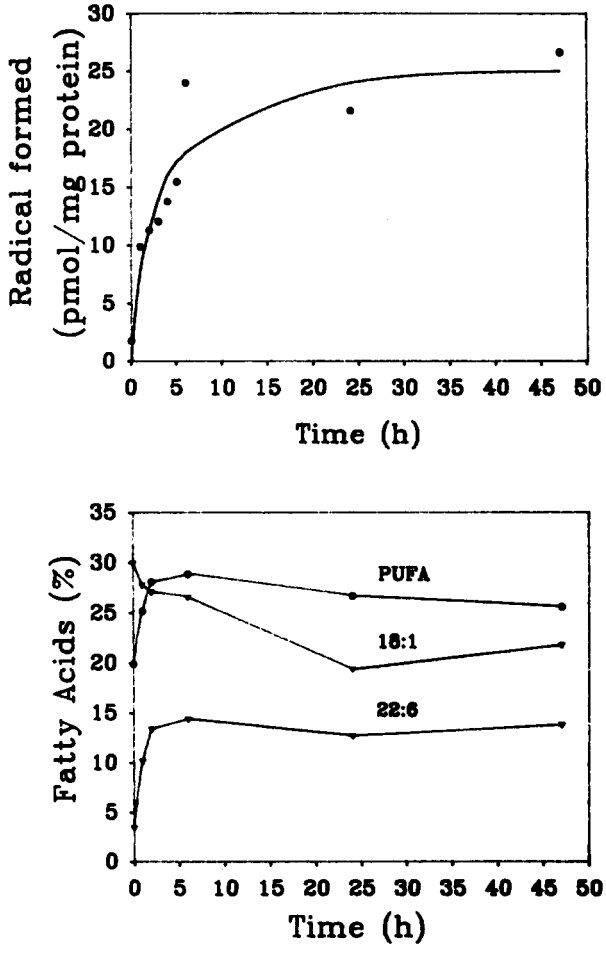

Fig. 5. Time dependence of radical adduct formation and fatty acid compositional changes after exposure of the endothelial cells to media supplemented with DHA. The conditions were the same as described in Fig. 1, except that the time of incubation of the PPAEC with a medium containing 20 μM DHA was varied. The cell fatty acid compositions were measured by GLC, but only the values for DHA (22:6), oleic acid (18:1), and total polyunsaturated fatty acids (PUFA) are shown. Each point represents a separate PPAEC culture. Similar results were obtained when this experiment was repeated with a different passage of PPAEC.

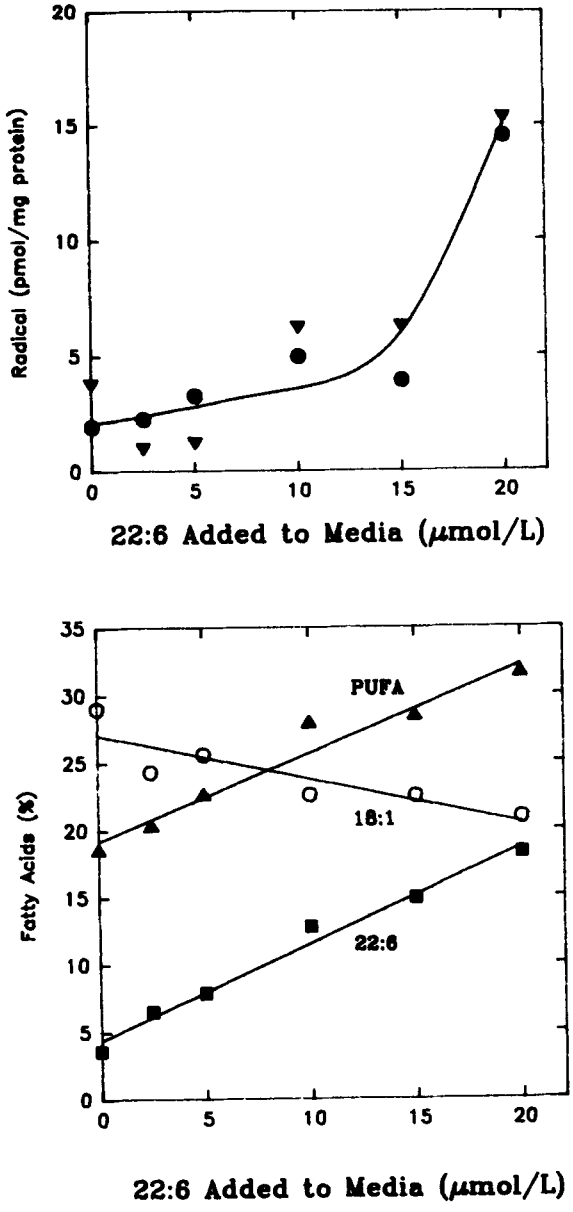

Fig. 6. Effect of DHA concentration on radical adduct formation and fatty acid composition. The conditions were the same as described in Fig. 1, except that the DHA concentration of the growth medium during the 24 h supplementation period was varied. Each point represents a separate PPAEC culture. The EPR segment of this study was done with two separate cell preparations. The results from the first experiment are represented by the circles; those from the second by the inverted triangles. The fatty acid compositional data are presented as described in Fig. 5.

68 ± 3.3 and 71 ± 2.0 nmol with the PPAEC, and 53 ± 5.8 and 39 ± 4.0 nmol with the BAEC. While these data suggest that a small reduction in lipid peroxidation may occur if POBN is present when endothelial cells are subjected to oxidant stress, the results are not conclusive because of the considerable variability in the data.

Effects of other polyunsaturated fatty acids

The comparative results obtained with DHA and EPA supplementation (Fig. 2) suggested that exposure of the endothelial cells to other polyunsaturated fatty acids might also enhance radical formation. Therefore, cells grown in media supplemented with different polyunsaturated fatty acids were tested. As shown in **Fig. 7,** spin adducts were detected when the PPAEC were supplemented with arachidonic acid (Fig. 7B), α-linolenic acid (Fig. 7C), γ-linolenic acid (Fig. 7D), and linoleic acid (Fig. 7E). The intensity of radical adduct signals in these cases appeared to be smaller than when the cells were enriched with EPA (Fig. 7A). This difference was confirmed by measuring the net increase in spin adduct formation in PPAEC supplemented with EPA, arachidonic, or α-linolenic acids, using 3-CP as the standard for the quantitative assay. The cells supplemented with EPA produced 24.6 pmol radical/mg protein, as compared with 10.8 and 10.7 pmol, respectively, in those supplemented with arachidonic or α-linolenic acids.

The fatty acid compositional changes that occurred in these PPAEC are shown in **Table 3.** Data from PPAEC grown in unsupplemented medium and medium supplemented with DHA are included for comparison. The main changes observed were: oleic acid supplementation, 60% increase in 18:1 and decreases in 16:0 and 18:0; linoleic acid supplementation, increase in 18:2, decrease in 18:1; α-linolenic acid supplementation, increases in 18:3 and 20:5, decrease in 18:1; γ-linolenic acid supplementation, increases in 20:3, 20:4 and 22:4, decrease in 18:1; and EPA supplementation, increases in 20:5 and 22:5, decrease in 18:1.

Fig. 7. EPR spectra of POBN radical adducts produced by PPAEC supplemented with different polyunsaturated fatty acids. The conditions were the same as described in Fig. 1, except that different fatty acids (15 μM) were added to the growth medium for 24 h. The fatty acids used were: A: EPA; B: arachidonic acid; C: α-linoleic acid; D: γ-linolenic acid; E: linoleic acid.

TABLE 3. Fatty acid composition of PPAEC grown on microcarrier beads with various fatty acid supplements

Fatty Acid	Fatty Acid Composition of Cell Lipids							
	Unsupplemented (n = 6)[a]	Oleic[b]	Linoleic	α-Linolenic	γ-Linolenic	Arachidonic	Eicosapentaenoic (n = 4)	Docosahexaenoic (n = 7)
					%			
16:0	20.2 ± 0.2	15.4	18.1	19.6	21.1	19.2	19.6 ± 0.7	22.1 ± 0.5
16:1	7.2 ± 0.4	2.5	2.6	4.6	3.5	3.3	4.1 ± 0.2	4.9 ± 0.4
18:0	14.9 ± 0.3	10.4	11.6	12.7	12.3	12.2	12.5 ± 0.3	13.9 ± 0.5
18:1	30.0 ± 0.7	47.9	19.7	20	18.8	17.1	22.0 ± 1.3	21.6 ± 0.6
18:2	1.7 ± 0.1	0.7	15.1	1.1	0.7	1.1	1.2 ± 0.1	1.3 ± 0.1
18:3	1.0 ± 0.2	nd	nd	7.3[c]	2.5[d]	nd	0.2 ± 0.1	0.9 ± 0.1
20:3	1.2 ± 0.1	0.9	4.7	1.1	13.4	1.2	1.1 ± 0.0	1.1 ± 0.1
20:4	6.1 ± 0.2	5.2	7.7	7.4	9.7	18.6	7.0 ± 0.3	5.0 ± 0.1
20:5	0.5 ± 0.1	0.9	0.2	3.1	nd	nd	4.4 ± 0.5	0.8 ± 0.2
22:4	2.1 ± 0.2	nd	3.9	1.7	4.3	15.6	2.1 ± 0.2	1.8 ± 0.3
22:5	1.8 ± 0.1	1.2	1.5	2.6	1.5	1.8	11.3 ± 1.5	1.6 ± 0.1
22:6	3.4 ± 0.4	1.3	1.9	1.5	2.1	1.3	1.7 ± 0.2	14.3 ± 0.5
Other	6.1	7.4	7.5	13.6	7.0	5.7	7.7	7.0
Saturated	36.9 ± 0.5	27.8	31.6	34.3	35.8	33.6	35.4 ± 1.1	38.0 ± 0.8
Monounsaturated	38.4 ± 0.8	52.7	23.1	25.5	22.8	21.0	26.9 ± 1.3	26.5 ± 0.8
Polyunsaturated	17.7 ± 0.7	12.0	37.8	26.3	34.2	39.6	30.3 ± 1.6	28.0 ± 0.5
Carbons[e]	16.08 ± 0.16	17.84	18.12	17.86	18.2	18.64	18.0 ± 0.14	17.89 ± 0.06
Double bonds[f]	1.53 ± 0.02	1.07	1.52	1.48	1.59	1.91	1.72 ± 0.07	1.72 ± 0.03

PPAEC were grown for 2 days either in unsupplemented medium or medium containing 15 μmol/l supplemental fatty acid. Results are expressed as the mean ± standard error, except in those cases where only one culture was analyzed. Values do not total 100% because only those fatty acids comprising > 3% of the total or those exhibiting appreciable changes are included. For the fatty acid saturation classes, average chain length, and average number of double bonds calculations, all of the fatty acids listed above are included, as well as peaks identified as 14:0, 14:1, 20:2, 22:0, 22:1, 24:0, and 24:1. nd, not detected.

[a] Number of individual cultures analyzed.

[b] Supplemental fatty acid added to the growth medium.

[c] 18:3 n-3.

[d] 18:3 n-6.

[e] Average chain length of fatty acyl chains.

[f] Average number of double bonds per fatty acyl chain.

Reversibility of DHA-induced response

Experiments were done to determine whether the increased response to oxidant stress resulting from enrichment with DHA was reversible. After 24 h of enrichment with 20 μM DHA, the PPAEC were washed and subdivided into two groups. One group was continued in the DHA-supplemented medium, and the other was transferred to a medium in which the DHA supplement was replaced with either 5 or 10 μM oleic acid. After 18 h, these media were removed, the cell-coated beads were washed, and EPR spectra were recorded after addition of POBN and $FeSO_4$. **Figure 8** shows that, as compared with the cells maintained in DHA (Fig. 8A), the radical adduct signal was reduced after transfer to the medium containing 10 μM supplemental oleic acid (Fig. 8C). However, there was no reduction in those cells transferred to 5 μM oleic acid (Fig. 8B).

GLC analysis of the cell lipids was done to determine whether transfer to oleic acid resulted in any changes in fatty acid composition. The control cultures, which continued in the medium originally supplemented with DHA, contained 10.4% 22:6 and 22.7% 18:1. The cul-

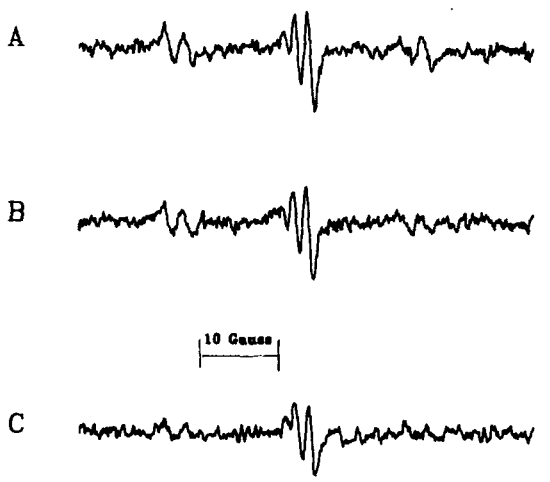

Fig. 8. Incubation with oleic acid reduces radical adduct formation in cells previously supplemented with DHA. PPAEC were supplemented with DHA for 24 h as described in Fig. 1. After this incubation, the medium was removed and the beads containing the cells were incubated for an additional 18 h with fresh medium containing 20 μM DHA (A), 5 μM oleic acid (B), or 10 μM oleic acid (C) as a supplement. After removal of these media, the beads were suspended in chelating resin-treated phosphate buffer containing POBN and $FeSO_4$, and EPR spectra were obtained as described in Fig. 1.

tures transferred to 5 μM oleic acid contained 10.2% 22:6 and 23.5% 18:1. The cells transferred to 10 μM oleic acid contained 8.1% 22:6 and 26.6% 18:1. Therefore, 5 μM supplemental oleic acid is not sufficient to alter the fatty acid composition of the cells appreciably, consistent with the finding that it did not reduce the response of the DHA-enriched cells to oxidative stress. By contrast, exposure to 10 μM oleic acid reduced the DHA content, and this was enough to decrease the intensity of radical formation.

To further explore reversibility, a second transfer experiment was done with DHA-enriched PPAEC. After a 24-h incubation in media containing 20 μM DHA, the cultures were washed and divided into two groups. The EPR response in cultures from each group was measured by exposure to POBN and $FeSO_4$ at the time of transfer. One set of cultures was maintained in the medium containing DHA, while the other was transferred to a medium containing 20 μM oleic acid. Cultures from each group were washed and tested for radical formation at 6 or 22 h after the transfer. As seen in **Fig. 9**, the amount of radical adduct formed by the cells continuously exposed to DHA, as measured by signal averaging (27), increased slightly during the 22-h test period. Conversely, radical adduct formation decreased when the DHA-supplemented cultures were transferred to the medium containing 20 μM oleic acid; some reduction was apparent within 6 h, and a further decrease occurred after 22 h.

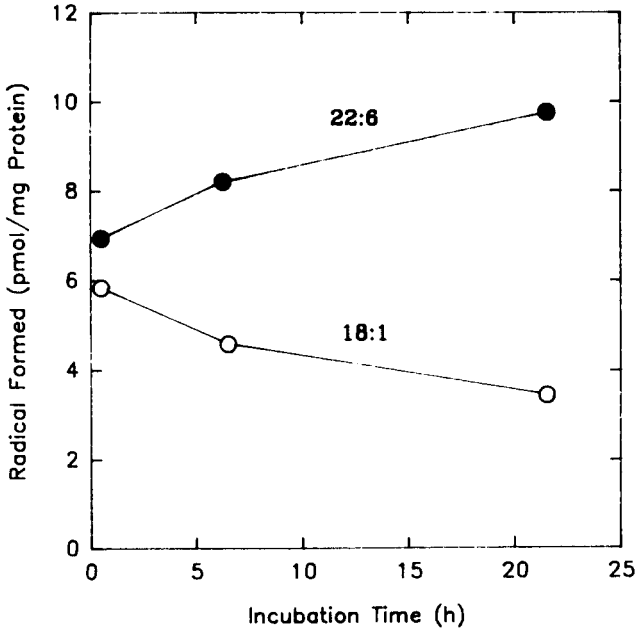

Fig. 9. Time dependence of reversibility of radical adduct formation in cells previously enriched with DHA. The PPAEC initially were enriched for 24 h with 20 μM DHA. After this medium was removed and the cultures were washed, they were transferred to media containing 20 μM DHA (22:6), or 20 μM oleic acid (18:1) as a supplement. EPR signal intensity was measured within 15–40 min after transfer, 6 h after transfer, and 22 h after transfer, using 3-CP as the standard. The EPR measurements were made by signal averaging (27) in 0.5 ml chelating resin-treated phosphate buffer, 25 mM POBN, and 100 μM $FeSO_4$.

TABLE 4. Reversibility of lipid radical adduct formation in U937 monocytes

Cell Treatment	Number of Cultures	Radical Adducts Formed
		pmol/mg protein
Continuous in DHA	5[a]	30.3 ± 5.6
Transferred to oleic acid	6	13.8 ± 3.2[b]

All cultures were grown in medium supplemented with 20 μM DHA. This medium was removed after 2 days and the cells were washed by sedimentation. In one set of cultures, 8×10^6 cells were resuspended in 0.5 ml chelating resin-treated buffer containing 10 mM POBN; 100 μM $FeSO_4$ was added, and the mixture was transferred to a flat cell for EPR measurements. In the other set, the cells were resuspended in a medium supplemented with 20 μM oleic acid. After 18 h, this medium was removed, the cells were washed, and EPR measurements were made on 8×10^6 cells as described above. Radical adduct formation was calculated by comparing the signal intensities with those obtained from a series of 3-CP standards.

[a] One culture in this set was lost.

[b] $P < 0.01$.

Because we have only a limited capacity to prepare separate spinner cultures, we could not test a large number of endothelial-microcarrier bead preparations in a single experiment. Therefore, the reproducibility of the oleic acid effect could not be evaluated statistically in the endothelial system. To overcome this, we carried out similar reversibility studies with U937 monocytes, a human transformed cell line that grows in suspension and produces POBN radical adducts when exposed to $FeSO_4$ (13). **Table 4** shows that the U937 cells supplemented continuously with 20 μM DHA produced 1.5- to 4-times more radical adducts in response to Fe^{2+} than the endothelial cells (compare this value with those in Figs. 5, 6, and 9). As in the case of the endothelial cultures, however, transfer for 18 h to a medium containing 20 μM oleic acid reduced radical adduct formation in the U937 cells by 55% ($P < 0.01$).

DISCUSSION

These findings indicate that similar to malignant U937 monocytes (13), aortic and pulmonary artery endothelial cells produce POBN radical adducts when they are exposed to oxidant stress. Therefore, the production of lipid radicals in amounts sufficient to be detected by EPR is not limited to transformed cells in suspension; it also occurs in adherent, nonmalignant vascular cells.

As in the case of the U937 cells, the characteristics of the EPR spectrum produced by endothelial cells are consistent with the formation of a short-chain alkyl radical derived by cleavage of oxidized polyunsaturated fatty acyl chains (14, 15). We suggest that this results from lipid peroxidation, initiated by the abstraction of a H atom

from a methylene bridge in a polyunsaturated fatty acyl chain (11, 28-30). This is accomplished in our experimental system by the addition of iron, a transition metal (31-34). Previous work with U937 cells demonstrated that addition of $FeSO_4$ generates oxidants similar to a hydroxyl radical, OH •, which could be the actual initiating agent (13). The lipid radical that is formed reacts rapidly with O_2 (11, 35), producing the lipid peroxyl radical, LOO•. This is the chain-carrying intermediate of the peroxidation cycle. The LOOH produced can lead to additional branching events by forming the alkoxyl radical, LO•. The latter undergoes β-scission, producing aldehydes and short-chain alkyl radicals. Based on a comparison of the EPR spectral characteristics of the spin adduct with those generated in chemical and enzymatic peroxidation systems (14, 15), we suggest that POBN traps some of the short-chain alkyl radicals that are formed. These probably are ethyl, pentyl, or pentenyl fragments derived from the polyunsaturated fatty acids that accumulate in the intracellular lipids when the cells are grown in a fatty acid-supplemented medium. Thus, the trapped radicals are secondary products formed after the initial polyunsaturated fatty acid radical reacts with O_2.

An alternative possibility is that the fatty acid supplements contained oxidized material, such as fatty acid hydroperoxides, that were taken up by the cells. This would seed the endothelial lipids with material that is readily oxidizable by $FeSO_4$. However, we did not obtain spectral evidence for the presence of conjugated dienes in the DHA preparation, even at a concentration almost 10^3-times higher than that present in the culture media. Radioactive oxidation products also were not detected in the incubation media containing [1-^{14}C]DHA (Fig. 4B and C). However, a small amount of a more polar radioactive product, which could be an oxygenated form of DHA, was detected in the cells during incubation with [1-^{14}C]DHA (Fig. 4D-F). Therefore, the possibility that an oxidation product was generated intracellularly from the added DHA and that this triggered radical formation when the cells were exposed to $FeSO_4$ cannot be completely excluded.

Five spin traps were tested in this work, but radical adducts were detected only with POBN. It is not clear why the process should be specific for only this spin trap. One possibility is that, of the spin traps used, only POBN was able to enter the location where the secondary radicals are formed. In interpreting these findings, however, one must realize that we tested the four other spin traps only under conditions that gave positive results with POBN, and it is possible that each spin trap may require a quite different set of experimental conditions to act effectively with intact cells.

The TBARS data indicate that the presence of POBN does not appreciably inhibit cellular lipid peroxidation in response to oxidant stress. This is consistent with our interpretation that POBN traps a secondary radical generated at the end of the peroxidation cycle. While the presence of POBN may have caused a small decrease in TBARS production, the values are too variable to be conclusive. Therefore, based on the present results, we cannot tell whether trapping some of the secondary radicals may significantly reduce the extent of lipid peroxidation. The magnitude of TBARS formation was about 10^4-times larger than the amount of POBN radical adducts detected, but these values cannot be strictly compared because the EPR spectra were obtained during the first 6-7 min after the $FeSO_4$ was added, whereas the TBARS reaction required a 1-h incubation in order to form enough material for a fluorescence measurement. However, the large disparity in the values suggests that the magnitude of radical adduct formation detected by this assay may not be an ideal quantitative estimate of the amount of lipid peroxidation occurring in the cells. On the other hand, TBARS not only arise from lipids, but also from proteins, carbohydrates, and nucleic acids when tissues are subjected to free radical oxidation, so it is likely to overestimate the lipid response.

A comparison of the values in Table 4 with those in Figs. 5, 6, and 9 indicates that, after DHA enrichment, the U937 cells produce about twice as much POBN radical adducts as the endothelial cells. An observation that could explain this finding is that the U937 cells accumulate more DHA than the endothelial cells under these conditions of supplementation. For example, when the growth medium contains 10 μM DHA, 22:6 accounts for 13% of the PPAEC fatty acids (Fig. 6, bottom), whereas it comprises 27% of the U937 cell fatty acids (13). This is consistent with increased formation of radicals in the U937 cells. It may be related to the fact that the U937 cells are neoplastic or that they are in suspension, whereas the endothelial cells are attached to a surface of a microcarrier bead during the spin trapping assay.

The fatty acid compositional changes resulting from supplementation of the endothelial cells grown on beads with DHA or other n-3 and n-6 polyunsaturated fatty acids are similar to those observed when polyunsaturated fatty acids are added to endothelial monolayers growing in tissue culture plates (36-40). For example, like the monolayers (37, 38), the endothelial cells attached to microcarrier beads elongate α-linolenic acid and EPA, but they do not convert measurable amounts of these n-3 fatty acids to DHA. Since radical adduct formation was enhanced by α-linolenic acid and EPA, as well as by n-6 polyunsaturated fatty acids, the process is not specific for either DHA or the n-3 class of polyunsaturates. Rather, it appears to be a nonspecific lipid peroxidation process that can utilize both of the commonly occurring classes of polyunsaturated fatty acids.

The concentration dependence data demonstrate that the response to oxidant stress increases abruptly when the DHA added to the growth medium was raised from 15–20 μM. A corresponding abrupt change in the fatty acid composition of the cells did not occur; this continued to change linearly as the DHA concentration was raised (Fig. 6). Apparently, a critical level of unsaturation is reached with respect to a response to oxidant stress when the DHA content exceeds 13–15% of the cell fatty acids, even though there is no abrupt shift in fatty acid composition. The increased responsiveness may be due to clustering of polyunsaturated fatty acyl chains as the content increases, a process that would be expected to facilitate the propagation reaction of lipid peroxidation. Alternatively, the increase in polyunsaturated fatty acid substrate available for the initiation and propagation phases of lipid peroxidation may exceed the antioxidant capacity of the endothelial cells when the degree of unsaturation reaches a critical level.

The time-dependent studies indicate that the increased response to oxidant stress begins to take effect shortly after the cells are exposed to elevated amounts of DHA. Likewise, the increased responsiveness begins to subside relatively soon after the DHA supplement is removed, indicating that the process can be reversed fairly quickly. These findings suggest that the susceptibility of the vascular endothelium to lipid peroxidation may be responsive to changes in the composition of rapidly turning over plasma fatty acid pools, such as free fatty acid or triglycerides contained in chylomicrons and very low density lipoproteins. As opposed to polyunsaturated fatty acids, supplementation with oleic acid reduced the responsiveness of the endothelial cells to oxidant stress. Furthermore, transfer of the cells from a medium enriched with DHA to one containing supplemental oleic acid led to some reduction in the sensitivity to Fe^{2+}-induced lipid radical formation within 6 h. These results are consistent with previous findings that supplementation with *cis*-vaccenic acid, an 18-carbon monounsaturated fatty acid, protected PPAEC against oxidant injury induced by hyperoxia or hydrogen peroxide (41, 42). Based on the present data, it would appear that this monounsaturated fatty acid probably replaced some of the inherent polyunsaturates in the PPAEC, thereby reducing the sensitivity to oxidant stress.

Studies by Reaven et al. (43, 44) have shown that plasma low density lipoproteins obtained from humans fed an oleic acid-enriched diet are less susceptible to copper-mediated oxidation than those from subjects fed a linoleic acid-rich diet. This potentially beneficial effect is one reason why, in attempting to reduce plasma low density lipoprotein levels, it may be preferable to replace dietary saturated fat with monounsaturated fat, as opposed to polyunsaturated fat (43–46). The present results dealing with cells derived from the arterial wall are entirely consistent with this general concept. They suggest that, as in the case of low density lipoproteins, chronic exposure to oleic acid supplements will make the endothelium less responsive to a given level of oxidant stress, as compared with supplements containing either n-3 or n-6 polyunsaturated fatty acids. Furthermore, they suggest that under certain conditions, a reduction in endothelial sensitivity to oxidant stress will begin to occur fairly rapidly if elevated levels of polyunsaturated fatty acids subside and are replaced by oleic acid. Thus, monounsaturated fatty acids may have beneficial effects for both lipoproteins and the vascular wall in terms of protection against oxidative injury.

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REFERENCES

1. Hennig, B., and C. K. Chow. 1988. Lipid peroxidation and endothelial cell injury: implications in atherosclerosis. *Free Radical Biol. Med.* **4:** 99–106.
2. Rosen, G. M., and B. A. Freeman. 1984. Detection of superoxide generated by endothelial cells. *Proc. Natl. Acad. Sci. USA.* **81:** 7269–7273.
3. Zweir, J. L., P. Kuppusamy, and G. A. Lutty. 1988. Measurements of an endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues. *Proc. Natl. Acad. Sci. USA.* **85:** 4046–4050.
4. Weiss, S. J., J. Young, A. F. LoBuglio, A. Slivka, and N. F. Nimel. 1981. Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J. Clin. Invest.* **68:** 714–721.
5. Shasby, D. M., S. E. Lind, S. S. Shasby, J. C. Goldsmith, and G. W. Hunninghake. 1985. Reversible oxidant-induced increases in albumin transfer across cultured endothelium: alteration in cell shape and calcium homeostasis. *Blood.* **65:** 605–614.
6. Shatos, M. A. 1992. Modulation of the fibrinolytic response of cultured human vascular endothelium by extracellularly generated oxygen radicals. *J. Biol. Chem.* **267:** 597–601.
7. Whorton, A. R., M. E. Montgomery, and R. S. Kent. 1985. Effect of hydrogen peroxide on prostaglandin production in cultured porcine aortic endothelial cells. *J. Clin. Invest.* **76:** 295–302.
8. Hempel, S. L., D. L. Haycraft, J. C. Hoak, and A. A. Spector. 1990. Reduced prostacyclin formation after reoxygenation of anoxic endothelium. *Am. J. Physiol.* **259:** C739–C745.
9. Britigan, B. E., T. L. Roeder, and D. M. Shasby. 1992. Insight into the nature and site of oxygen-centered free radical generation by endothelial cell monolayers using a novel spin trapping technique. *Blood.* **79:** 699–707.
10. Fox, R. B. 1984. Prevention of granulocyte-mediated ox-

idant lung injury in rats by a hydroxyl radical scavenger, dimethylthiourea. *J. Clin. Invest.* **74:** 1456-1464.
11. Buettner, G. R. 1993. The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* **300:** 535-543.
12. Harris, P., and P. Ralph. 1985. Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. *J. Leukocyte Biol.* **37:** 407-422.
13. North, J. A., A. A. Spector, and G. R. Buettner. 1992. Detection of lipid radicals by electron paramagnetic resonance spin trapping using intact cells enriched with polyunsaturated fatty acid. *J. Biol. Chem.* **267:** 5743-5746.
14. Chamulitrat, W., and R. P. Mason. 1990. Alkyl free radicals from the β-scission of alkoxyl free radicals as detected by spin trapping in a lipoxygenase system. *Arch. Biochem. Biophys.* **282:** 65-69.
15. Iwahashi, H., P. W. Albro, S. R. McGown, K. B. Tomer, and R. P. Mason. 1991. Isolation and identification of α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone radical adducts formed by the decomposition of the hydroperoxides of linoleic acid, linolenic acid, and arachidonic acid by soybean lipoxygenase. *Arch. Biochem. Biophys.* **284:** 172-180.
16. Kaduce, T. L., A. A. Spector, and R. S. Bar. 1982. Linoleic acid metabolism and prostaglandin production by cultured bovine pulmonary artery endothelial cells. *Arteriosclerosis.* **2:** 380-389.
17. Buettner, G. R. 1990. Use of ascorbate as test for catalytic metals in simple buffers. *Methods Enzymol.* **186:** 125-127.
18. Buettner, G. R. 1988. In the absence of catalytic metals ascorbate does not autooxidize at pH 7: ascorbate as a test for catalytic metals. *J. Biochem. Biophys. Methods.* **16:** 27-40.
19. Buettner, G. R., and K. P. Kiminyo. 1992. Optimal EPR detection of weak nitroxide spin adduct and ascorbyl free radical signals. *J. Biochem. Biophys. Methods.* **24:** 147-151.
20. Lees, M. B., and S. Paxman. 1972. Modification of the Lowry procedure for the analysis of proteolipid protein. *Anal. Chem.* **47:** 184-192.
21. Folch, J., M. B. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497-509.
22. Kaduce, T. K., A. A. Spector, and G. E. Folk. 1980. Characterization of the plasma lipids and lipoproteins of the polar bear. *Comp. Biochem. Biophys.* **69B:** 541-545.
23. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.* **5:** 600-608.
24. Yagi, K. 1984. Assay for blood plasma or serum. *Methods Enzymol.* **105:** 328-331.
25. Asakawa, T., and S. Matsushita. 1980. Coloring conditions of thiobarbituric acid test for detecting lipid hydroperoxides. *Lipids.* **15:** 137-140.
26. Denning, G. M., P. H. Figard, T. L. Kaduce, and A. A. Spector. 1983. Role of triglycerides in endothelial cell arachidonic acid metabolism. *J. Lipid Res.* **24:** 993-1001.
27. Buettner, G. R., E. E. Kelley, and C. P. Burns. 1993. Membrane lipid free radicals produced from L1210 murine leukemia cells by Photofrin photosensization: an electron paramagnetic resonance spin trapping study. *Cancer Res.* **53:** 3670-3673.
28. Porter, N. 1984. Chemistry of lipid peroxidation. *Methods Enzymol.* **105:** 273-282.
29. Gardner, H. W. 1989. Oxygen radical chemistry of polyunsaturated fatty acids. *Free Radical Biol. Med.* **7:** 65-86.
30. Barclay, L. R. C. 1993. Model membranes: quantitative studies of peroxidation, antioxidant action, partitioning, and oxidative stress. *Can. J. Chem.* **71:** 1-16.
31. Miller, D. M., G. R. Buettner, and S. D. Aust. 1990. Transition metals as catalysts of "autoxidation" reactions. *Free Radical Biol. Med.* **8:** 95-108.
32. Brieland, J. K., S. J. Clarke, S. Karmiol, S. H. Phan, and J. C. Fantone. 1992. Transferrin: a potential source of iron for oxygen free radical-mediated endothelial injury. *Arch. Biochem. Biophys.* **294:** 265-270.
33. Reif, D. W. 1992. Ferritin as a source of iron for oxidative damage. *Free Radical Biol. Med.* **12:** 417-427.
34. Yin, D., H. Lingnert, B. Ekstrand, and U. T. Brunk. 1992. Fenton reagents may not initiate lipid peroxidation in an emulsified linoleic acid model system. *Free Radical Biol. Med.* **13:** 543-556.
35. Hasegawa, K., and L. K. Patterson. 1978. Pulse radiolysis studies in model lipid systems: formation and behavior of peroxy radicals in fatty acids. *Photochem. Photobiol.* **28:** 817-823.
36. Spector, A. A., J. C. Hoak, G. L. Fry, G. M. Denning, L. L. Stoll, and J. B. Smith. 1980. Effect of fatty acid modification on prostacyclin production by cultured human endothelial cells. *J. Clin. Invest.* **65:** 1003-1012.
37. Spector, A. A., T. L. Kaduce, J. C. Hoak, and G. L. Fry. 1981. Utilization of arachidonic and linoleic acids by cultured human endothelial cells. *J. Clin. Invest.* **68:** 1003-1011.
38. Spector, A. A., T. L. Kaduce, P. H. Figard, K. C. Norton, J. C. Hoak, and R. L. Czervionke. 1983. Eicosapentaenoic acid and prostacyclin production by cultured human endothelial cells. *J. Lipid Res.* **24:** 1595-1604.
39. Hadjiagapiou, C., T. L. Kaduce, and A. A. Spector. 1986. Eicosapentaenoic acid utilization by bovine aortic endothelial cells: effects on prostacyclin production. *Biochim. Biophys. Acta.* **875:** 369-381.
40. Vossen, R. C. R. M., M. A. H. Feijge, J. W. M. Heemskerk, M. C. E. van Dam-Mieras, G. Hornstra, and R. F. A. Zwaal. 1993. Long-term fatty acid modification of endothelial cells: implications for arachidonic acid distribution in phospholipid classes. *J. Lipid Res.* **34:** 409-420.
41. Hart, C. M., J. K. Tolson, and E. R. Block. 1990. Fatty acid supplementation protects pulmonary artery endothelial cells from oxidant injury. *Am. J. Respir. Cell. Mol. Biol.* **3:** 479-489.
42. Hart, C. M., J. K. Tolson, and E. R. Block. 1992. Quantitative fatty acid analyses in cultured porcine pulmonary artery endothelial cells: the combined effects of fatty acid supplementation and oxidant exposure. *J. Cell. Physiol.* **153:** 76-87.
43. Reaven, P. D., S. Parthasarathy, B. J. Grasse, E. Miller, F. Almazan, F. H. Mattson, J. C. Khoo, D. Steinberg, and J. Witztum. 1991. Feasibility of using an oleic acid-rich diet to reduce the susceptibility of low density lipoprotein to oxidative modification in humans. *Am. J. Clin. Nutr.* **54:** 701-706.
44. Reaven, P., S. Parthasarathy, B. J. Grasse, E. Miller, D. Steinberg, and J. Witztum. 1993. Effects of oleic acid-rich and linoleic acid-rich diets on the susceptibility of low density lipoprotein to oxidative modification in mildly hypercholesterolemic subjects. *J. Clin. Invest.* **91:** 668-676.
45. Mattson, F. H., and S. M. Grundy. 1985. Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. *J. Lipid Res.* **26:** 194-202.
46. Grundy, S. M., and M. A. Denke. 1990. Dietary influences on serum lipids and lipoproteins. *J. Lipid Res.* **31:** 1149-1172.