Enrichment of LDL with EPA and DHA Decreased Oxidized LDL-Induced Apoptosis in U937 Cells[*]

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Wu, T., Geigerman, C., Lee, Y.-S., and Wander, R.C. 2002. Enrichment of LDL with eicosapentaenoic acid and docosahexaenoic acid decreased oxidized LDL-induced apoptosis in U937 cells. Lipids. 37(8):789-96.

Abstract:

Oxidized LDL (oxLDL) may contribute to the accumulation of apoptotic cells in atherosclerotic plaques. Although it is well established in monophasic chemical systems that the highly unsaturated EPA and DHA will oxidize more readily than FA that contain fewer double bonds, our previous studies showed that enrichment of LDL, which has discrete polar and nonpolar phases, with these FA did not increase oxidation. The objective of this study was to compare the extent of apoptosis induced by EPA/DHA-rich oxLDL to that induced by EPA/DHAnon-rich oxLDL in U937 cells. LDL was obtained from one healthy subject three times before and after supplementation for 5 wk with 15 g/d of fish oil (FO), an amount easily obtainable from a diet that contains fatty fish. After supplementation, an EPA/DHA-rich LDL was obtained. Oxidative susceptibility of LDL, as determined by measuring the formation of conjugated dienes and the accumulation of cholesteryl ester hydroperoxides, was not higher in EPA/DHA-rich LDL. The oxLDL-induced cell apoptosis was detected by the activation of caspase-3, the translocation of PS to the outer surface of the plasma membrane using the Annexin V-fluorescein isothiocyanate binding assay, and the presence of chromatin condensation and nuclear fragmentation using the 4,6-diamidino-2-phenylindole staining assay. All three measures showed that after FO supplementation, EPA/DHA-rich oxLDL-induced cell apoptosis decreased. The decrease was not related to the concentration of lipid hydroperoxides. This study suggests that a possible protective effect of EPA/DHA-rich diets on atherosclerosis may be through lessening cell apoptosis in the arterial wall.

Paper no. L8943 in Lipids 37, 789–796 (August 2002).

Article:

Recent findings show that apoptosis is responsible for much of the cell death seen during atherosclerosis. Indeed, apoptosis has been positively correlated with the localization and severity of atherosclerosis (1). Macrophages, smooth muscle cells, and lymphocytes undergoing apoptosis all have been found in human atherosclerotic lesions (2–4). Of the three cell types, macrophages appear to be the preferred cell population in atherosclerotic lesions to undergo

[*] Abbreviations: AMC, aminomethyl coumarin; CE18:200H, cholesteryl linoleate hydroperoxide; CEOOH, cholesteryl ester hydroperoxides; DAPI, 4,6- diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; FO, fish oil; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; nLDL, native LDL; oxLDL, oxidized low-density lipoprotein; PI, propidium iodide.

apoptosis (2,5,6). Apoptotic macrophages have been implicated in the initiation and enlargement of the cellular lipid-core in the advanced atherosclerotic lesion (7–9). Furthermore, this process can lead to the activation of thrombosis (7–9).

Oxidized LDL (oxLDL) plays a critical role in the development of atherosclerosis (10,11). Recently, oxLDL has been shown to induce apoptosis in human umbilical vein endothelial cells (12,13), smooth muscle cells and fibroblasts (14), and macrophages (15,16).

It is conceivable that the role of oxLDL in the development of atherosclerosis could be through induction of apoptosis. The mechanisms by which oxLDL induce apoptosis are not well understood. Lipid hydroperoxides (17), decomposition products (i.e., 4-hydroxynonenal) (18), and cholesterol oxidation products (oxysterols) (i.e., 25-hydroxycholesterol or 7-ß hydroxycholesterol) (13,19–21) are generated during oxidation of LDL and have been shown to induce apoptosis. However, the relative potencies of these different products are not yet known. Furthermore, upregulated Fas and Fasligand (22), the release of cytochrome c from mitochondria (21), the activation of caspase-8 (22), caspase-3 (13,15,21), and an increase in cellular calcium concentrations (23) have all been implicated in oxLDL-induced cell apoptosis.

Current research has attempted to identify those factors that regulate the oxidation of LDL and the production of its decomposition products. It is believed that an improved understanding of these factors may provide a therapeutic tool for controlling the development and progression of atherosclerosis. For example, dietary FA influence the rate and extent of LDL oxidation. EPA and DHA are FA mainly from fish that are associated with a decreased risk of cardiovascular disease. In a recent clinical trial, results showed that enriching LDL with EPA/DHA produced a lower concentration of lipid hydroperoxides compared to the LDL enriched with linoleic acid (24), when LDL was subjected to oxidation. Given that lipid hydroperoxides are the predominant lipid oxidation products found in atherosclerotic lesions (25), our findings support a role of EPA/DHA in reducing the severity of atherosclerosis. Moreover, reduced production of lipid hydroperoxides may lessen the formation of secondary oxidation products such as 4-hydroxynonenal, thereby reducing apoptosis.

The effect of oxidized LDL that has been enriched with EPA/DHA on cell apoptosis has not been investigated. In order to evaluate this, the oxidative susceptibility of EPA/DHA-rich LDL was compared to that of EPA/DHA-nonrich LDL. The extent of apoptosis of U937 cells induced by
EPA/DHA-rich oxLDL was compared to that of EPA/DHAnon-rich oxLDL. U937 cells are human pro-monocytic cells and share many characteristics with macrophages. Additionally, they are one of the easiest cell models in which to study apoptosis. EPA/DHA-rich oxLDL contained less or equivalent concentrations of lipid hydroperoxides as did EPA/DHAnon-rich oxLDL. Apoptosis of U937 cells was induced after 6–7 h of incubation with oxLDL, and was lower in cells treated with EPA/DHA-rich oxLDL compared to cells treated with EPA/DHA-non-rich oxLDL.

MATERIALS AND METHODS

Supplementation and experimental design

The University of North Carolina at Greensboro Institutional Review Board approved the current study, and the subject signed informed consent prior to entering the study. A healthy, nonsmoking male (37 yr) was supplemented with 15 g/d fish oil (FO) for 5 wk. Plasma was collected on three separate days before and after supplementation. The FA profile of the oil was measured by GC using heptadecanoic acid (Nu-Chek-Prep, Elysian, MN) as an internal standard as previously described (26). The supplement (15 g) provided 1.26 g EPA and 1.08 g DHA. This amount (2.34 g) of n-3 FA is similar to that found in two servings (200 g) of Chinook salmon (27). FO supplements were kindly provided by OmegaPure™ (Houston, TX) in bulk and were then encapsulated by Banner Pharmacaps, Inc. (High Point, NC). A mixture of α-, γ-, and δ-tocopherols (courtesy of ADM Nutraceuticals, Decatur, IL) and TBHQ (Eastman Chemical Company, Kingsport, TN) were added to the oil. The vitamin E content of the oil was measured by HPLC (Agilent Technologies 1100 series, Palo Alto, CA) using a fluorometric detector set at 292 nm for excitation and 330 nm for emission (28). Recovery of α-tocopherol added to the sample was 92%, and the interassay coefficient of variation was 5.6%. After these additions, the oil contained 0.540 mg α-tocopherol/g oil, 0.689 mg γ-tocopherol/g oil, 0.177 mg δ-tocopherol/g oil, and 0.02% TBHQ.

Cell culture and treatment

A week before the experiments, U937 cells (ATCC, Rockville, MD) were thawed from the frozen vials and maintained at 1–5 × 10^5/mL cells in T-75 culture flasks in RPMI 1640 medium supplemented with 10% FBS, 1% glutamine (200 mmol/L), 1% penicillin-streptomycin (10,000 IU/mL), and 0.2% amphotericin B (250 µg/mL) at 37°C in a 95% air/5% CO_2 humidified incubator. These cells were used in subsequent experiments. Preparation of lipoproteins and determination of LDL oxidative susceptibility. Blood samples were collected into Vacutainer® tubes (Beckton Dickinson, Franklin Lakes, NJ) containing Na_2EDTA (1 g/L) after an overnight fast (≥10 h). Plasma was immediately isolated by centrifugation at 1200 × g for 15 min at 4°C (Jouan GR 4-12, Winchester, VA). An aliquot of plasma was stored at −80°C for later measurement of the lipid profile. The lipid profile, including plasma concentrations of cholesterol, triacylglycerol (TAG), HDL-cholesterol (HDL-C), and LDL-cholesterol (LDL-C), were measured to ensure that the subject was normolipemic. Plasma concentrations of cholesterol and TAG were measured as previously described (29). The cholesterol concentration of the HDL fraction was measured by an enzymatic method in samples after the precipitation of the LDL and VLDL fractions with phosphotungstic acid in conjunction with $MgCl_2$ (Sigma Kit #352-4, St. Louis, MO) (30). LDL-C concentration was calculated using the following formula: total cholesterol − HDL-C
– TAG/5 (31). LDL was isolated from plasma by centrifugation using a modification of the method of Chung et al. (32) as modified by Chen and Loo (33). Briefly, 28 mL of NaCl (0.195 M) was carefully overlaid on 11.5 mL of plasma in a 39.5 mL tube (Beckman, Quick-seal, Palo Alto, CA). The ultracentrifuge tube was placed in a 50.2 Ti rotor (Beckman) and centrifuged at 190,000 × g (40,000 rpm) for 2 h in a Beckman L7-65 ultracentrifuge.

An aliquot of the isolated LDL was stored at −80°C for measurement of the FA profile. The FA profile was measured by GC as previously described (26) using heptadecanoic acid (NuChek-Prep) as an internal standard. Following removal of EDTA and KBr from a second aliquot of LDL by using a 10 DG disposable column (Bio-Rad Laboratories, Hercules, CA), the cholesterol

concentration was determined enzymatically as previously described (29) and is referred to as LDL-C.

The EDTA/KBr-free LDL was oxidized; and cholesteryl ester hydroperoxide (CEOOH) concentration, apoptotic events, and conjugated diene formation were measured. CEOOH and apoptosis were measured in aliquots taken from the same oxidized solution. In this system 0.4 mg LDL-C/mL was oxidized with 5 μM Cu^{2+}. The formation of conjugated dienes was measured in a separate system by continuously monitoring their production at 234 nm in a Beckman DU-640 spectrophotometer, as we have previously reported (34), at 37°C over a 6-h time period. The concentration of conjugated dienes was calculated using the molar extinction coefficient 29,500 $M^{-1}cm^{-1}$ (35). The three variables—lag time, rate of oxidation, and maximal concentration of conjugated dienes— were determined. Maximal rate was determined to be the slope of the line of best fit through the points that defined the steepest slope of the curve. The length of the lag phase was determined to be the time (value for x) at the intersection of the line describing the maximal rate and the line describing the initial rate. The maximal concentration was determined from the maximum absorption measured during the 6-h oxidation. The absorption at 234 nm plateaued at about 5 h and then began to decrease, indicating that secondary oxidation products were being formed as the conjugated dienes degraded. As many products are formed during this time and their absorptivity is unknown, monitoring the absorbance at 234 nm would yield ambiguous results and was terminated at 6 h. The maximal rate of formation and the concentration of conjugated dienes were expressed relative to the protein content of the EDTA/KBr-free LDL. The protein content of the LDL was determined using a kit obtained from Bio-Rad Laboratories. It was previously determined that optimal performance for the measurement of conjugated dienes was obtained when 55 μg LDL protein/mL (0.25 mg LDL/mL) was used (34). However, to make comparisons between the system in which CEOOH and apoptosis were measured and that in which conjugated dienes were measured, the ratio of Cu^{2+} to LDL-C had to be constant. This was accomplished by measuring the protein content of the EDTA/KBr-free LDL and diluting it to 55 μg protein/mL. Because the dilution factor was known, the concentration of cholesterol in this diluted solution could be calculated. Once the cholesterol concentration was known, the concentration of $CuSO_4$ needed to maintain consistency across both systems could be calculated. This calculation was made for each sample.

To measure CEOOH concentration and apoptosis in the EDTA/KBr-free LDL sample before oxidation was initiated by the addition of $CuSO_4$, a sample of LDL was removed and EDTA (1.5 mg/mL) was immediately added to prevent oxidation. This sample is identified as native LDL (nLDL). The remaining EDTA/KBr-free LDL was oxidized. An aliquot was removed after 6 h, and EDTA (1.5 mg/mL) was added immediately to halt the oxidation reactions. The remaining EDTA/KBr-free LDL sample was oxidized four more hours (a total of 10 h), and again EDTA was added to terminate the oxidation reactions. HPLC with an isoluminol-dependent chemiluminescence detection method, as previously reported (24), with slight modification was used to analyze nLDL, 6-h oxLDL, and 10-h oxLDL for CEOOH formation. Briefly, nLDL and oxLDL were extracted with hexane, evaporated, and dissolved in ethanol. Samples were injected onto a C-18 column (Supelcosil LC-18, 5 μm, 250 × 4.6 mm; Supelco, Bellefonte, PA) using a mobile phase of methanol/tertbutanol (1:1, vol/vol) at a flow rate of 1.0 mL/min. A postcolumn chemiluminescence reagent consisting of 3:7 (vol/vol) methanol to 100 mM sodium borate buffer (pH 10) containing 1 mM isoluminol (6-amino-2,3-dihydro-1,4-

phthalazinedione; Sigma), and 3 mg/L microperoxidase (MP-11; Sigma) at a flow rate of 1.5 mL/min was added to the eluent. Cholesteryl linoleate hydroperoxide (CE 18:2OOH) (Cayman Chemicals, Ann Arbor, MI) was used as an external standard to quantify the amount of CE18:2OOH present in the samples. The modifications gave a single peak from CEOOH rather than several peaks, each associated with a different CEOOH, as reported previously (24). Thus, a more global assessment of the oxidation of core lipids was obtained.

Apoptosis assays

Apoptosis was assessed by three methods: caspase-3 activity, translocation of PS, and condensed chromatin and nuclear fragmentation. In the caspase-3 method, acetyl-asp-glu-val-asp-aminomethyl coumarin (Ac-DEVDAMC), a synthetic tetrapeptide fluorogenic substrate, was added to the samples. This substrate is cleaved in the presence of caspase-3, releasing the fluorescent AMC moiety, which can be quantified by fluorescence intensity spectrophotometrically (36) (PharMingen #6332K, San Diego, CA). For the second method, translocation of PS from the inner to the outer leaflet of the plasma membrane was measured by the Annexin V binding assay. In this assay Annexin V conjugated to fluorescein isothiocyanate (FITC) binds to the translocated PS, and the amount of the conjugate can be quantified fluorometrically. This assay can be used as a marker of early apoptosis (37). In addition, in this assay cells were costained with propidium iodide (PI). This is used as a marker for cell membrane permeability, a change that occurs during the latter stages of apoptosis and in necrosis. Viable and early apoptotic cells, which have intact membranes, exclude PI, but necrotic cells do not. Apoptotic cells are FITC-positive and PI-negative, whereas necrotic cells are both FITC- and PI-positive. In the third assay, condensed chromatin and nuclear fragmentation were visualized microscopically after staining the cells with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI).

All three measures of apoptosis were made before and after supplementation with FO. Each treatment was performed in triplicate. The cells were incubated with unoxidized LDL (nLDL), LDL that was oxidized for 6 h (6-h oxLDL), and LDL that was oxidized for 10 h (10-h oxLDL). The concentration of LDL-C was maintained at 66 μg/mL for caspase-3 activity and DAPI staining measurements and 133 μg/mL for PS translocation. Incubations with the LDL were 6–7 h long. All experiments included positive and negative controls. For the negative control, cells were incubated in LDL-free medium. Two positive controls were used. One positive control was to treat the cells with 25-hydroxycholesterol (62.5–66.7 μg/mL) (Sigma-Aldrich, St. Louis, MO) for 6 h. The other one was to treat them
with (1.5 μg/mL) camptothecin (Sigma-Aldrich) for 4 h. A shorter incubation was used for the camptothecin because it induced apoptosis more quickly. Since the supplementation lasted about 5 wk, the LDL that was enriched with EPA/DHA was obtained later than the LDL that was not enriched with these FA. The use of these two positive controls allowed us to establish that the U937 cells behaved in a similar fashion at both times.

For the measurement of caspase-3 activity, 5×10^5 cells were incubated with the negative and two positive controls, nLDL, 6-h oxLDL, and 10-h oxLDL. Cells were harvested and washed 2× with PBS (pH 7.4, 10 mmol/L), resuspended in lysis buffer (100 μL buffer/L × 10^6 cells), and stored at −20°C for batch analysis. Samples were analyzed using a fluorescence

spectrophotometer (PerkinElmer LS 5OB, Norwalk, CT) set at 380 nm for excitation and 420 nm for emission. Data are expressed as relative fluorescent intensity per 10^5 cells.

For the measurement of translocation of PS, 2.5×10^5 cells were incubated in the same six systems: the negative and positive controls, nLDL, 6-h oxLDL, and 10-h oxLDL. After incubation, 0.3 mL of the cell suspension was removed from each well and washed 2× with ice-cold PBS (Ca^{2+}:Mg 2+-
free). The resulting cell pellet was suspended in 0.1 mL binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/LNaCl, 2.5 mmol/L $CaCl_2$], 5 µL of Annexin V-FITC (as supplied by PharMingen #65874x, San Diego, CA), and 10 µL of PI stock solution (50 µg/mL) and then incubated in the dark for 15 min. After incubation, an additional 0.4 mL aliquot of binding buffer was added to the cell solution. Cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) using the FL1 channel to detect the signal generated by Annexin V-FITC and the FL2 channel to detect the signal generated by PI. Data are expressed as the percentage of cells positively stained with Annexin V-FITC but negatively stained with PI.

For the DAPI staining assay 2.5 x 10^5 cells were incubated with native LDL, 10-h oxLDL, and the controls. Cells were harvested and washed 2x with PBS (pH 7.4, 10 mmol/L) before being resuspended in 200 µL of PBS and loaded on poly-Llysine slides (Sigma #P-0425) in Cytospin Chambers (StatSpin, Inc. #CC03, Norwood, MA) for 10 min. Slides were cytospun in a Cytofuge®2 (StatSpin, Inc.) for 4 min at 850 rpm (40 x g), immersed in fresh 3.7% formaldehyde solution for 20 min at 4°C, and subsequently stored at least one night in ice-cold 70% ethanol at —20°C. Following storage, slides were washed 3x with PBS, immersed into a diluted DAPI solution (1 µg/mL) for 10 min, and then viewed on an Olympus BX-60 fluorescence microscope (Melville, NY) equipped with a SPOT digital camera (Sterling Heights, MI). The excitation wavelength was 358 nm, and the emission wavelength was 461 nm. The apoptotic cells were identified by a bright blue nucleus, characteristic of either condensed or fragmented chromatin, whereas normal cells were characterized by a faint blue nucleus. At least 80 cells were counted from three randomly selected fields on each slide. Slides were examined for each sample. The percentage of apoptotic cells was calculated by dividing the number of apoptotic cells by the total number of cells x 100%.

Statistical analysis

The significance of supplementation with FO on the lipid profile, the FA profile, and LDL oxidative susceptibility was determined using a two-sample t-test (38). The repeated measure's procedure was used to determine statistically significant differences between time points as well as the FO supplements on CEOOH concentration (38). To determine the influence of the length of time that LDL was oxidized on the extent of apoptosis, the data were analyzed using a one-way ANOVA (38). When a significant difference was found, pair-wise comparisons were performed using the Tukey–Kramer test to determine significant differences among treatments (38). To determine the effect of FO supplementation on extent of apoptosis, a two-sample t-test was used (38). All experiments were repeated three times before and after supplementation. Analyses were completed using the SAS general linear model procedure (version 8; SAS Institute Inc., Cary, NC). A value of $P < 0.05$ was considered significant.

RESULTS

The TAG concentration in plasma was reduced ($P = 0.02$) from baseline by 51% after FO supplementation. The FO supplementation did not significantly ($P > 0.05$) alter the concentration of cholesterol, HDL-C, or calculated cholesterol in LDL. The LDL FA profile changed after supplementation with FO (Fig. 1). The concentration of EPA (20:5n-3) and DHA (22:6n3) in LDL was 1100 and 110% higher after supplementation, respectively, compared to baseline ($P < 0.05$ for both FA).

The lag time and maximal rate of production for conjugated dienes were significantly ($P < 0.05$) different from baseline after FO supplementation (Table 1). The lag time was reduced by 32 min, and the maximal rate of production for conjugated dienes was reduced by 37%. However, the maximal amount of conjugated dienes produced in the LDL was similar ($P > 0.05$) after FO supplementation compared to baseline.

The concentration of CEOOH in LDL both before and after supplementation with fish oil, i.e., the concentration in nLDL, was less than 180 pmol/mg LDL-C, the lower limit of detection of this assay (Fig. 2). The concentration of CEOOH produced in LDL oxidized for 6 h was 35% lower ($P = 0.007$) after FO supplementation than before FO supplementation. In contrast, the concentration of CEOOH in the LDL before and after FO supplementation was statistically equivalent in the samples oxidized for 10 h ($P > 0.05$) (Fig. 2).

For all measures of apoptosis, the response seen in the negative controls, i.e., those cells incubated in LDL-free medium (Fig. 3), was similar to the response seen in U937 cells treated with nLDL, suggesting that LDL did not initiate apoptosis. There were no significant differences ($P > 0.05$) before and after FO supplementation for U937 cells treated with either 25-hydroxycholesterol or camptothecin, regardless of which measure of apoptosis was used. This suggests that the apoptotic response of the U937 cells was similar even when measures were separated by the 5-wk supplementation interval.

Two statistical assessments of the data were performed. One was to determine if the extent of apoptosis was influenced by the length of time that LDL was oxidized. This comparison was made separately for the samples obtained before and after supplementation with FO. The second evaluation was to determine if the supplementation with FO influenced the extent of apoptosis induced by LDL after the LDL was oxidized for a defined time.

Influence of length of time that LDL was oxidized on extent of apoptosis

For all three measures of apoptosis, oxidation of LDL increased the extent of apoptosis (Fig. 3). Both before and after supplementation with FO, the activity of caspase-3 was higher in cells treated with LDL oxidized for 6 or 10 h compared to cells treated with nLDL ($P = 0.0001$ in all comparisons except for cells treated with LDL oxidized for 6 h after FO supplementation, where it was 0.0324). However, although

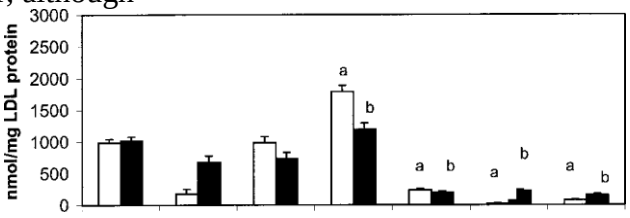

FIG. 1. FA concentration in LDL before (open bars) and after (solid bars) supplementation with fish oil. Data are least square means (LSM) ± SEM. Bars with different letters above them for each FA are significantly different, P<_ 0.05.

Table 1: Lag Time, Rate of Formation of Conjugated Dienes, and Maximum Production of Conjugated Dienes in LDL
Before and After Supplementation with Fish Oil'
Maximum rate

	Lag time (min)	(nmol/mg LD protein/min)	Maximum production (nmol/mg LDL protein)
Before FO	140 ± 2	1.87 ± 0.08	186 ± 17
After FO	108 ± 2	1.17 ± 0.08	171 ± 17
Before FO vs. after FO	0.0002	0.0032	0.5749

[a]Least square means (LSM) ± SEM calculated for triplicate determinations made from three independent experiments. bP value from two-sample t-test.

the activity of caspase-3 was higher in the cells treated with LDL oxidized for 10 h compared to those treated with LDL oxidized for 6 h before supplementation with FO (P = 0.0001), its activity was statistically equivalent in those cells treated with LDL oxidized for 6 and 10 h after supplementation with FO.

In using the Annexin V assay, both before and after supplementation with FO, the percentage of apoptotic cells was higher in cells treated with LDL oxidized for 6 and 10 h (P = 0.0001) compared to cells treated with nLDL. However, in contrast to the caspase-3 assay, the extent of apoptosis was significantly higher in the cells treated with LDL oxidized for 10 h compared to LDL oxidized for 6 h both before (P = 0.0001) and after FO supplementation (P = 0.0033).
In using the DAPI staining assay both before and after FO supplementation, the percentage of apoptotic cells was higher in cells treated by LDL oxidized for 10 h compared to cells treated with nLDL (P = 0.0001).

Influence of FO supplementation on extent of apoptosis

Apoptosis induced by oxLDL, regardless of assay used, was decreased by FO supplementation. The activity of caspase-3 in U937 cells treated with LDL oxidized for 6 and 10 h after supplementation with FO was reduced by 13 (P = 0.0053) and 22% (P = 0.0001), respectively, compared to treatment with LDL oxidized for the same length of time but obtained before

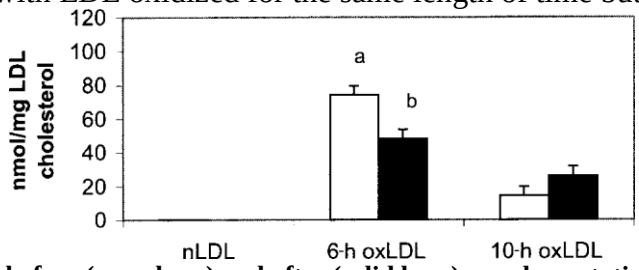

FIG. 2. Lipid peroxidation before (open bars) and after (solid bars) supplementation with fish oil as indicated by the concentration of cholesteryl ester hydroperoxides in LDL. Each of the labels on the x-axis are defined as follows: nLDL, native LDL, is the LDL sample before any oxidation has occurred; 6-h oxLDL represents LDL oxidized for 6 h with copper; 10-h oxLDL represents LDL oxidized for 10 h. Data are least square means (LSM) ± SEM. Bars with different letters above them at each time for LDL oxidation are significantly different, P<_ 0.05.

supplementation with FO (Fig. 3A). On the basis of the Annexin V assays, only after the LDL had been oxidized for 10 h was a significant difference measured in the extent of apoptosis before and after FO supplementation. It was 30% lower ($P < 0.0001$) in U937 cells treated with EPA/DHA-rich LDL compared to EPA/DHA-non-rich LDL oxidized for 10 h (Fig. 3B). Results from the DAPI staining assays showed a 61% reduction ($P = 0.0001$) in apoptosis for U937 cells treated with LDL oxidized for 10 h after FO supplementation compared to before supplementation (Fig. 3C).

DISCUSSION

Apoptotic vascular cells have been found in atherosclerotic lesions (2–4). Since oxLDL has been reported to induce apoptosis of endothelial cells (12,13), smooth muscle cells and fibroblasts (14), and macrophages (15,16) in vitro, it may be a candidate for the induction of apoptosis of vascular cells in vivo. The oxidized lipids in oxLDL are thought to be responsible for apoptosis. Thus, events that modify the bioactive lipid profile of oxLDL may affect its ability to induce apoptosis. We showed in this study and previously (24,34) that LDL enriched with EPA and DHA has the intuitively predicted shorter lag time, suggesting increased oxidative susceptibility, but a paradoxical decrease in rate of formation of conjugated dienes, suggesting decreased oxidative susceptibility. The explanation that we have offered is that bicycloendoperoxides are formed in the presence of EPA and DHA and that they rapidly migrate to the surface of the LDL particle because of their polarity. Thus, since radicals are removed from the system, the rate of the propagation is slowed. These reactions would alter the lipid entities present in oxLDL that would induce apoptosis. Thus, the purpose of this study was to determine if EPA/DHA-rich oxLDL, obtained after FO supplementation, altered the extent of apoptosis as compared to the LDL obtained prior to FO supplementation. Several standard apoptotic markers were used to provide a better confirmation of apoptosis. The results showed that oxLDL induced apoptosis in U937 cells, as characterized by the activation of caspase-3, translocation of PS, and chromatin condensation and nuclear fragmentation. However, LDL obtained after supplementation with FO and then oxidized induced less apoptosis than LDL obtained before supplementation with FO.

LDL enriched with EPA/DHA and oxidized for 10 h induced significantly less apoptosis in U937 cells by all three measures used. However, when the U937 cells were treated with LDL oxidized for 6 h, the decrease was significant only when measured by caspase-3 activity. Caspase-3 causes the proteolysis of several substrates (39) and thereby brings about the characteristic morphological changes seen in apoptosis, such as the alteration of the cellular membranes and nuclei (40). Thus, the activation of caspase-3 occurs before the translocation of PS, chromatin condensation, or nuclear fragmentation. That the EPA/DHA-rich LDL oxidized for a shorter period of time (6 h) did not cause significant differences in the changes of the translocation of PS suggests that the changes in translocation of PS induced by the presence of these FA may require the activation of caspase-3 and occur after the activation of caspase-3 (41). It does not eliminate the possibility that other pathways are also associated with the translocation of PS and are triggered by oxLDL containing EPA/DHA. The release of cytochrome c from mitochondria (21), the activation of caspase-8 (22) and caspase-3 (13,15,21), and an increase in cellular calcium concentrations (23) all have been implicated in oxLDL-induced cell apoptosis. Whether they work together with caspase-3 or independently is not known.

Although the extent of apoptosis was higher in the cells treated with LDL oxidized for 10 h than LDL oxidized for 6 h, the concentration of CEOOH was higher in the LDL oxidized for 6 h than

that in LDL oxidized for 10 h. This divergence suggests that CEOOH were not the causative agents. In this study, the concentration of conjugated dienes reached its maximum value after approximately 5 h of oxidation. It is well-established (35) that by the time the concentration of conjugated dienes plateaus, secondary oxidation products have begun to form. However, since secondary oxidation products were not measured in this study, it cannot be concluded that they were the causative factor.

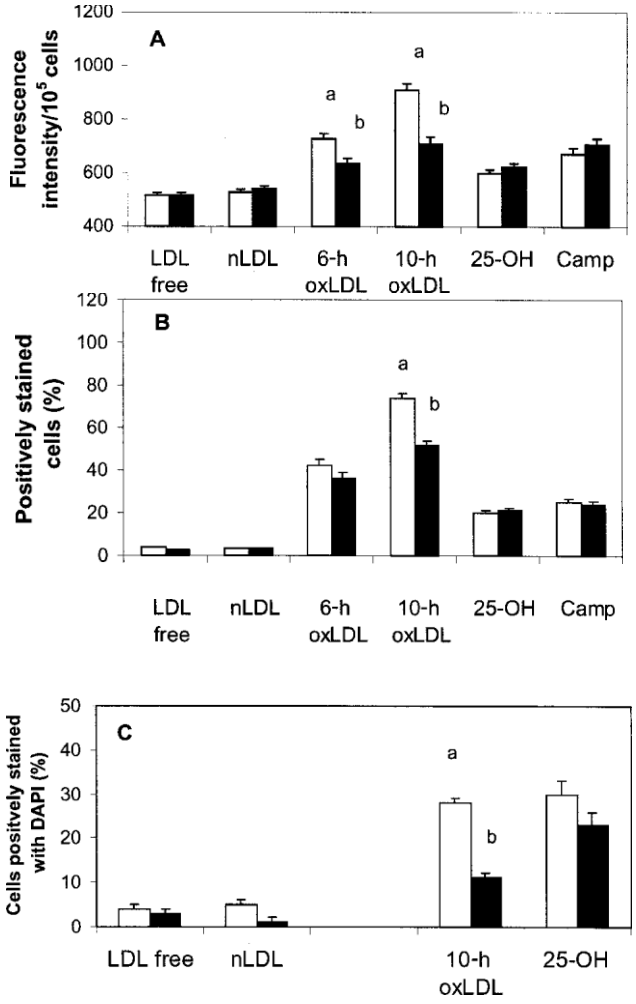

FIG. 3. Measurement of apoptosis in U937 cells induced by LDL. Cells were incubated with nLDL; 6-h oxLDL; and 10-h oxLDL. Cells were incubated without LDL (free LDL) as a negative control. They were incubated with 25-hydroxycholesterol (25-OH) or camptothecin (Camp) as positive controls. Two statistical assessments of the data were performed. One was to determine if the extent of apoptosis was influenced by the length of time that the LDL was oxidized. This comparison is discussed in the text. The second evaluation was to determine if the supplementation with fish oil influenced the extent of apoptosis induced by LDL oxidized for a defined length of oxidation (open bars, before supplementation with fish oil; solid bars, after supplementation). The results from this comparison are represented here. (A) Measurement of activity of caspase-3. The activity of caspase-3 was expressed as fluorescence intensity per 105 cells. (B) Measurement of U937 cells positively stained with Annexin V–fluorescein isothiocyanate (FITC), negatively stained with

propidium iodide. Data are displayed as the percentage of 104 cells stained with Annexin VFITC. (C) Measurement of 4,6-diamidino-2-phenylindole (DAPI) staining of U937 cells. Apoptotic cells were characterized by a bright blue nucleus and condensed and fragmented chromatin. Data are least square means (LSM) ± SEM. Bars with different letters above them have a significant effect produced by the fish oil supplementation, P<_ 0.05. For other abbreviations see Figure 2.

The suggestion that the CEOOH generated by oxLDL were not responsible for mediating cell apoptosis is indirectly supported by Lizard et al. (19) and Harada-Shiba et al. (13). Both of these groups of investigators showed that oxysterols induced apoptosis in smooth muscle and endothelial cells. Liu et al. (18) also showed that a different form of secondary products, i.e., 4-hydroxynonenal, induced apoptosis in smooth muscle cells. However, Siow et al. (14) reported more apoptotic events in smooth muscle cells at the point of greatest lipid hydroperoxide production compared with secondary lipid oxidation products, i.e., aldehydes and ketones. The divergence among the above findings may reflect differences in the degree of LDL oxidation and/or the fact that a thorough evaluation of secondary oxidation products was not done.
A strength of this study is that the concentration of lipid hydroperoxides, CEOOH, was measured directly via HPLC with an isoluminol-dependent chemiluminescence detection method. This is among the most sensitive methods for detecting lipid hydroperoxides directly, rather than their presence being inferred, as is done with other measures. It clearly showed CEOOH was not the major compound responsible for apoptosis.

In conclusion, we have demonstrated that enrichment of oxLDL with EPA/DHA, obtained after supplementing a human with FO, protected U937 cells against oxLDL-induced apoptosis and was not related to the concentration of CEOOH. However, the oxLDL used in these studies may not simulate oxLDL in vivo. Thus, the relevance of the data collected in this study to explain the bioactivity of oxLDL in vivo
is unknown. Although copper is frequently used to mediate LDL oxidation, it may not be a physiologically relevant initiator for LDL oxidation. LDL oxidized ex vivo with copper may provide insight into the impact on the oxidation of LDL and vascular walls in vivo that occurs when individuals consume diets rich in EPA and DHA. This study suggests that an increased concentration of EPA/DHA in LDL may influence its oxidative susceptibility and subsequent pathology and that the major compounds responsible for apoptosis are not CEOOH.

ACKNOWLEDGMENTS
This study was supported in part by the North Carolina Agriculture Experimental Station and the Linus Pauling Institute (Corvallis, OR). We would like thank Dr. George Loo in the Department of Nutrition and Foodservice Systems at the University of North Carolina at Greensboro and Dr. Mark L. Failla in the Department of Human Nutrition and Foodservice Management at the Ohio State University for their invaluable advice.

REFERENCES

1. Stehbens, W.E. (2000) The Significance of Programmed Cell Death or Apoptosis and Matrix Vesicles in Atherogenesis, Cell. Mol. Biol. 46, 99–110.
2. Björkerud, S., and Björkerud, B. (1996) Apoptosis Is Abundant in Human Atherosclerotic Lesions, Especially in Inflammatory Cells (macrophages and T cells), and May Contribute to the Accumulation of Gruel and Plaque Instability, Am. J. Pathol. 149, 367–380.

3. Han, D.K.M., Haudenschild, C.C., Hong, M.K., Tinkle, B.T., Leon, M.B., and Liau, G. (1995) Evidence for Apoptosis in Human Atherogenesis and in a Rat Vascular Injury Model, Am. J. Pathol. 147, 267–277.
4. Mitchinson, M.J., Hardwick, S.J., and Bennett, M.R. (1996) Cell Death in Atherosclerotic Plaques, Curr. Opin. Lipidol. 7, 324–329.
5. Hegyi, L., Skepper, J.N., Cary, N.R., and Mitchinson, M.J. (1996) Foam Cell Apoptosis and the Development of the Lipid Core of Human Atherosclerosis, J. Pathol. 180: 423–429.
6. Isner, J.M., Kearney, M., Bortman, S., and Passeri, J. (1995) Apoptosis in Human Atherosclerosis and Restenosis, Circulation 91, 2703–2711.
7. Ball, R.Y., Stowers, E.C., Burton, J.H., Cary, N.R., Skepper, J.N., and Mitchinson, M.J. (1995) Evidence That the Death of Macrophage Foam Cells Contributes to the Lipid Core of Atheroma, Atherosclerosis 114: 45–54.
8. Hegyi, L., Hardwick, S.J., Siow, R.C., and Skepper, J.N. (2001) Macrophage Death and the Role of Apoptosis in Human Atherosclerosis, J. Hematother. Stem Cell. Res. 10, 27–42.
9. Kockx, M.M., and Knaapen, M.W. (2000) The Role of Apoptosis in Vascular Disease, J. Pathol. 190, 267–280.
10. Witztum, J.L., and Steinberg, D. (1991) Role of Oxidized LDL in Atherogenesis, J. Clin. Invest. 88, 1785–1792.
11. Berliner, J.A., and Heinecke, J.W. (1996) The Role of Oxidized Lipoproteins in Atherosclerosis, Free Radic. Biol. Med. 20, 707–727.
12. Dimmeler, S., Haendeler, J., Galle, J., and Zeiher, A.M. (1997) Oxidized Low-Density Lipoprotein Induces Apoptosis of Human Endothelial Cells by Activation of CPP32-like Pro- teases. A Mechanistic Clue to the ‘Response to Injury’ Hypothesis, Circulation 95, 1760–1763.
13. Harada-Shiba, M.K., Kinoshita, M.K., Kamido, H.S., and Shimokado, K.T. (1998) Oxidized Low Density Lipoprotein Induces Apoptosis in Cultured Human Umbilical Vein Endothelial Cells by Common and Unique Mechanisms, J. Biol. Chem. 273, 9681–9687.
14. Siow, R.C., Richards, J.P., Pedley, K.C., Leake, D.S., and Mann, G.E. (1999) Vitamin C Protects Human Vascular Smooth Muscle Cells Against Apoptosis Induced by Moderately Oxidized LDL Containing High Levels of Lipid Hydroperoxides, Arte-rioscler. Thromb. Vasc. Biol. 19, 2387–2394.
15. Wintergerst, E.S., Jelk, J., Rahner, C., and Asmis, R. (2000) Apoptosis Induced by Oxidized Low Density Lipoprotein in Human Monocyte-Derived Macrophages Involves CD36 and Activation of Caspase-3, Eur. J. Biochem. 267, 6050–6059.
16. Hardwick, S.J., Hegyi, L., Clare, K., Law, N.S., Carpenter, K.L., Mitchinson, M.J., and Skepper, J.N. (1996) Apoptosis in Human Monocyte-Macrophages Exposed to Oxidized Low Density Lipoprotein, J. Pathol. 179, 294–302.
17. Wang, T.G., Gotoh, Y., Jennings, M.H., Rhoads, C.A., and Aw, T.Y. (2000) Lipid Hydroperoxide-Induced Apoptosis in Human Colonic CaCo-2 Cells Is Associated with an Early Loss of Cellular Redox Balance, FASEB J. 14, 1567–1576.
18. Liu, W., Kato, M., Akhand, A.A., Hayakawa, A., Suzuki, H., Miyata, T., Kurokawa, K., Hotta, Y. Ishikawa, N., and Nakashima, I. (2000) 4-Hydroxynonenal Induces a Cellular

Redox Status-Related Activation of the Caspase Cascade for Apoptotic Cell Death, J. Cell. Sci. 113, 635–641.
19. Lizard, G., Monier, S., Cordelet, C., Gesquiere, L., Deckert, V., Gueldry, S., Lagrost, L., and Gambert, P. (1999) Characterization and Comparison of the Mode of Cell Death, Apoptosis Versus Necrosis, Induced by 7P-Hydroxycholesterol and 7-Ketocholesterol in the Cells of the Vascular Wall, Arterioscler. Thromb. Vasc. Biol. 19, 1190–2000.
20. Spyridopoulos, I., Wischhusen, J., Rabenstein, B., Mayer, P., Axel, D.I., Frohlich, K.U., and Karsch, K.R. (2001) Alcohol Enhances Oxysterol-Induced Apoptosis in Human Endothelial Cells by a Calcium-Dependent Mechanism, Arterioscler. Thromb. Vasc. Biol. 21, 439–444.
21. Yang, L., and Sinensky, M.S. (2000) 25-Hydroxycholesterol Activates a Cytochrome c Release-Mediated Caspase Cascade, Biochem. Biophys. Res. Commun. 278, 557–563.
22. Lee, T., and Chau, L. (2001) Fas/Fas Ligand-Mediated Death Pathway Is Involved in OxLDL-Induced Apoptosis in Vascular Smooth Muscle Cells, Am. J. Cell. Physiol. 280, C709–C718.
23. Vieira, O., Escargueil-Blanc, I., Meilhac, O., Basile, J.P., Laranjinha, J., Almeida, L., Salvayre, R., and Negre-Salvayre, A. (1998) Effect of Dietary Phenolic Compounds on Apoptosis of Human Cultured Endothelial Cells Induced by Oxidized LDL. Br. J. Pharmacol. 123: 565–573.
24. Higdon, J.V., Du, S.H., Lee, Y.S., Wu, T., and Wander, R.C. (2001) Supplementation of Postmenopausal Women with Fish Oil Does Not Increase Overall Oxidation of LDL ex vivo Compared to Dietary Oils Rich in Oleate and Linoleate, J. Lipid Res. 42, 407–418.
25. Suarna, C., Dean, R.T., May, J., and Stocker, R. (1995) Human Atherosclerotic Plaque Contains Both Oxidized Lipids and Relatively Large Amounts of a-Tocopherol and Ascorbate, Arterioscler. Thromb. Vasc. Biol. 15, 1616–1624.
26. Song, J., and Wander, R.C. (1991) Effects of Dietary Selenium and Fish Oil (MaxEPA) on Arachidonic Acid Metabolism and Hemostatic Function in the Rat, J. Nutr. 121, 284–292.
27. Wander, R.C., and Patton, B.D. (1991) Lipids and Fatty Acids of Three Species of Northeast Pacific Finfish Harvested in Summer, Food Composition Anal. 4, 128–135.
28. Arnaud, J., Fortis, I., Blachier, S., Kia, D., and Favier, A. (1991) Simultaneous Determination of Retinol, a-Tocopherol and ß- Carotene in Serum by Isocratic High-Performance Liquid Chromatography, J. Chromatogr. 572, 103–116.
29. Wander, R.C., Du, S.H., and Thomas, D.R. (1998) Influence of Long-Chain Polyunsaturated Fatty Acids on Oxidation of Low Density Lipoprotein, Prostaglandins Leukotrienes Essent. Fatty Acids 59, 143–151.
30. Marz, W., and Gross, W. (1986) Analysis of Plasma Lipoproteins by Ultracentrifugation in a New Fixed Angle Rotor: Evaluation of a Phosphotungstic Acid/$MgCl_2$ Precipitation and a Quantitative Lipoprotein Electrophoresis Assay, Clin Chim. Acta 160, 1–18.
31. Friedewald, W.T., Levy, R.I., and Fredrickson, D.S. (1972) Estimation of the Concentration of Low-Density Lipoprotein Cholesterol in Plasma, Without Use of the Preparative Ultracentrifuge, Clin. Chem. 18, 499–502.
32. Chung, B.H., Segrest, J.P., Ray, M.J., Brunzell, J.D., Hokanson, J.E., Krauss, R.M., Beaudrie, K., and Cone, J.T. (1986) Single Vertical Spin Density Gradient Ultracentrifugation, Methods Enzymol. 128, 181–209.

33. Chen, C., and Loo, G. (1995) Cigarette Smoke Extract Inhibits Oxidative Modification of Low Density Lipoprotein, Atherosclerosis 112, 177–185.
34. Wander, R.C., Du, S.-H., Ketchum, S.O., and Rowe, K.E. (1996) Interaction of Vitamin E and Fish Oil on LDL Oxidation in Postmenopausal Women With and Without Hormone Replacement
Therapy, Am. J. Clin. Nutr. 63, 184–193.
35. Esterbauer, H., Striegl, G., Puhl, H., and Rotheneder, M. (1989) Continuous Monitoring of in vitro Oxidation of Human Low Density Lipoprotein, Free Radic. Res. Commun. 6, 67–75.
36. Stennicke, H.R., and Salvesen, G.S. (1997) Biochemical Characteristics of Caspases-3, -6, -7, and -8, J. Biol. Chem. 272, 25719–25723.
37. Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995) Early Redistribution of Plasma Membrane Phosphatidylserine Is a General Feature of Apoptosis Regardless of the Initiating Stimulus: Inhibition by Overexpression of Bcl-2 and Abl, J. Exp. Med. 182, 1545–1556.
38. Ramsey, F., and Schafer, D. (1999) Statistical Sleuth: A Course in Methods of Data Analysis, Duxbury Press, Boston.
39. Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P., and Flavell, R.A. (1998) Reduced Apoptosis and Cytochrome c-Mediated Caspase Activation in Mice Lacking Caspase 9, Cell 94, 325–337.
40. Song, Z., and Steller, H. (1999) Death by Design: Mechanism and Control of Apoptosis, Trends Cell Biol. 9, M49–M52.
41. Belloc, F., Belaud-Rotureau, M.A., Lavignolle, V., Bascans, E., Braz-Pereira, E., Durrieu, F., and Lacombe, F. (2000) Flow Cytometry Detection of Caspase 3 Activation in Preapoptotic Leukemic Cells, Cytometry 40, 151–160.