Reduced effect on apoptosis of 4-hydroxyhexenal and oxidized LDL enriched with n-3 fatty acids from postmenopausal women

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Abstract:

Background: Oxidized low-density lipoprotein (oxLDL) promotes apoptosis in atherosclerotic plaques in the vascular wall, a process mediated through its oxidized lipids. 4-Hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE), derived from oxidation of n-6 and n-3 fatty acids, respectively, are among the major oxidized products in oxLDL.

Hypothesis: This study hypothesized that eicosapentaenoic acid/docosahexaenoic acid (EPA/DHA)-rich versus linoleic acid-rich oxLDL obtained from postmenopausal women and HNE versus HHE differentially influence apoptosis in U937 cells.

Experimental Design: Thirty healthy postmenopausal women were supplemented with 14 g/day safflower oil (SO), 7 g/day of both fish oil and SO (low dose LFO) or 14 g/day fish oil (high dose HFO) for 5 weeks. Low-density lipoprotein, obtained after supplementation, was oxidized with 5 μM CuSO4 at 37°C for 6 h. The concentration of cholesteryl ester hydroperoxides (CEOOH) and conjugated dienes was measured in the oxidized LDL (oxLDL). U937 cells were incubated with the oxLDL, 10 μM of HHE, 7 μM of HHE plus 3 μM of HNE, 5 μM of both HHE and HNE or 10 μM of HNE and the extent of apoptosis measured three ways.

Results: The concentration of CEOOH and conjugated dienes in oxLDL did not differ among the three treatment groups. The percent of apoptotic cells was approximately 40% lower when incubated with oxLDL obtained from the HFO-supplemented group than the SO- supplemented group measured by both the Annexin V and the DNA fragmentation assays (P=.04 and .004, respectively). Apoptosis of U937 cells was significantly lower in cells incubated with 10 μM of HHE, and mixtures of HHE and HNE than the 10 μM HNE when measured by the Annexin V, DNA fragmentation and 4,6-diamidino-2-phenylindole (DAPI) staining.

Conclusions: These data suggest that the cardioprotective properties of n-3 fatty acids may derive in part from their less reactive oxidized lipid metabolites.

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Article:

1. *Introduction*

Although cardiovascular diseases (CVD) are often thought of primarily as a problem for males, they also occur in women. In 2000, CVD claimed the lives of more women than men [1]. Data from observational studies suggested that hormone replacement therapy (HRT) in postmenopausal women reduced the risk of CVD by 35–50% [2–4]. Because of this, HRT was routinely prescribed to postmenopausal women. However, two placebo-controlled clinical trials published in the summer of 2002 changed the view of this widespread treatment. The Heart and Estrogen/ Progestin Replacement Study II (HERS II) [5] found that HRT did not decrease the risk of CVD in women with previously diagnosed coronary artery disease (CAD). The Women's Health Initiative (WHI) trial [6], a primary prevention trial of HRT in postmenopausal women, also failed to demonstrate any benefit of HRT for the prevention of CAD. Indeed, the estrogen plus progestin arm of the study was terminated early because the women in this group demonstrated a statistically significant increase in breast cancer with the use of HRT. Despite the negative implications of the use of HRT, many women continue this therapy. Consequently, lifestyle modifications known to decrease CVD-related mortality are critically important to this group.

Fish consumption, presumably through the highly unsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is clearly cardioprotective [7–12]. The mechanism by which this occurs is not definitively resolved and is most likely multifactorial. A possible linkage may be through the effects of these fatty acids on apoptosis in the atherosclerotic plaque.

Atherosclerosis is the underlying condition involved in the pathogenesis of CVD. Apoptosis or programmed cell death is one of the processes that contribute to atherogenesis and plaque formation. Although various cell types undergoing apoptosis, that is, macrophages, smooth muscle cells and lymphocytes, have all been found in human atherosclerotic lesions [13–15], the dominant apoptotic cell population is macrophages. The impact of apoptosis of macrophages during the atherosclerotic process is ambiguous. Theoretically, it could lead to increased plaque stability due to decreased collagen breakdown [16]. However, a loss in the macrophage population could also diminish the scavenging efficiency of apoptotic bodies, resulting in increased plaque instability, rupture and thrombosis.

Oxidized low-density lipoprotein (oxLDL) is present in the atherosclerotic plaque [17] and is known to induce apoptosis in various cell types including smooth muscle cells, fibroblasts and monocytes/macrophages [18–21]. Numerous compounds found in oxLDL, including lipid hydroperoxides and several aldehydes, have been shown to cause apoptosis [22,23]. In a pilot study [24], we recently showed that oxLDL enriched in EPA and DHA induced less apoptosis in U937 cells, a human promonocytic cell line, than oxLDL enriched with linoleic acid. Further, our data suggested that this attenuated response was not associated with changes in the content of lipid hydroperoxides of the oxLDL.

The 4-hydroxyalkenals, 4-hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE), are the most abundant aldehydes present in many tissues, body fluids and also in atherosclerotic lesions [25–28]. Hydroxynonenal and HHE are generated during the peroxidation of n-6 polyunsaturated fatty acid (PUFA) (i.e., linoleic acid and arachidonic acid) and n-3 PUFA (i.e., EPA and DHA), respectively. The differential effect of these aldehydes on induction of apoptosis has been poorly explored [29]. Consequently, the purpose of this study was twofold: first, to confirm in a clinical trial with postmenopausal women using HRT that EPA/DHA-enriched oxLDL decreased apoptosis of U937 cells compared to linoleic acid-enriched oxLDL; second, to determine whether HNE and HHE differentially influenced apoptosis in U937 cells.

2. *Methods and materials*

2.1. **Subjects**

Thirty postmenopausal women, between 51 and 71 years of age, were recruited from the Piedmont Triad in North Carolina. They were healthy, nonsmoking, normolipidemic postmenopausal females who were using HRT. The subjects were willing to maintain a stable weight, refrain from eating fish and take no nutritional supplements except calcium and vitamin D during the course of the study. The study protocol was reviewed and approved by the Institutional Review Board at The University of North Carolina at Greensboro, and written consent was obtained from each subject prior to beginning the study.

Subjects were asked to keep 3-day dietary records during the supplementation period. The nutritional content of the diets was analyzed using Food Processor Plus (version 7.1; ESHA, Salem, OR). Blood samples were collected before and after supplementation after an overnight fast of >_10 h into tubes containing Na2EDTA (1.5 mg/ml plasma). Plasma was separated by low-speed centrifugation at 1300 xg for 15 min at 4°C. Samples were either analyzed immediately or appropriately aliquoted and stored at —80°C until processed.

2.2. **Supplementation**

Subjects were randomly assigned to one of the three supplementation groups. The safflower oil (SO) group was given 14 g/day of linoleic acid-rich SO, the low dose fish oil group (LFO) was given 7 g/day of both fish oil and SO, and the high dose of fish oil group (HFO) was given 14 g/day fish oil. Subjects took the oil supplements for 5 weeks. The SO was purchased in bulk from Arista Industries (Darien, CT) and the fish oil was kindly provided by Omega Protein (Houston, TX). Banner Pharmacaps (High Point, NC) encapsulated both oils.

The fatty acid profile of the oils was measured by gas chromatography using heptadecanoic acid (NuChek Prep, Elysian, MN) as an internal standard as described previously [30]. The intake of monounsaturated fatty acids (MUFA) provided by the supplements was similar in all three groups. The intake of saturated fatty acid (SFA), primarily palmitic acid, was 1.7 g in the SO group, 2.5 g in the LFO group and 3.4 g in the HFO group. There was no detectable amount of EPA and DHA provided by the SO supplement. The LFO supplement provided 0.59 g/day of EPA and 0.50 g/day of DHA while the HFO supplement provided twice these amounts (1.18 g/day of EPA and 1.00 g/day of DHA). The goal of the supplementation was to simulate intakes of EPA and DHA that could be achieved by the daily consumption of fatty fish. The LFO supplement provided amounts that could be obtained from approximately one serving of

Chinook salmon and the HFO supplement provided the amount that could be obtained from two servings [31].

The antioxidant content of the three supplements was matched. To accomplish this, the vitamin E content of the oils was measured by normal phase high-pressure liquid chromatography (HPLC) [32]. Appropriate amounts of a-, Sand g-tocopherol (courtesy of ADM Nutraceuticals, Decatur, IL) were added to both oils. After these additions, the oils contained 0.53–0.58 mg a-tocopherol/g oil, 0.65–0.69 mg y-tocopherol/g oil and 0.16–0.18 mg S-tocopherol/g oil. The concentration of tertiary butyl hydroquinone (TBHQ) in the SO was matched to that in the fish oil (0.02 wt.%, Eastman Chemical, Kingsport, TN).

2.3. **Analysis of plasma**

The plasma fatty acid profile was measured by gas chromatography as previously described by Song and Wander [30]. Heptadecanoic acid (NuChek Prep) was added as an internal standard. Plasma total cholesterol concentrations were determined enzymatically using a modification of the method of Allain et al. [33]. Plasma triacylglycerol concentrations were measured using a modification of the method of McGowan et al. [34]. Concentrations of a-tocopherol in the plasma were measured by reverse-phase HPLC using fluorometric detection (excitation X292; emission X330) adapted from the procedures of Arnaud et al. [35]. Plasma a-tocopherol concentration was expressed relative to the lipid content of plasma (atocopherol concentration divided by the sum of the concentration of total cholesterol and triacylglycerol).

2.4. **Preparation of LDL**

Low-density lipoprotein was isolated from plasma by single-spin discontinuous density gradient ultracentrifugation as described by Chung et al. [36], immediately after the blood was drawn. Briefly, plasma was adjusted to density of 1.30 g/ml by adding solid KBr. NaCl (0.195 M) was carefully overlaid on the plasma in a 39.5-ml tube (Beckman, Palo Alto, CA). Samples were centrifuged at 190,000xg for 2 h in a Beckman L7-65 ultracentrifuge using a 50.2 Ti rotor (Beckman). After centrifugation, LDL was removed from the tubes. EDTA and KBr were removed from the LDL using a 10 DG disposable column (Bio-Rad Laboratories, Hercules, CA). Low-density lipoprotein was diluted to a concentration of 0.5 mg LDL cholesterol/ml and oxidized using 5 µM CuSO4 at 37°C for 6 h. The concentration of CEOOH in oxLDL was determined by HPLC with a postcolumn enzymatic reaction using a chemiluminescence detector as previously described [24]. The maximum concentration of conjugated dienes was measured in a separate system of oxLDL by monitoring their continuous production at 37°C and 234 nm, as we have previously reported [24]. The cholesterol content of LDL was measured enzymatically by a modification of the method of Allain et al. [33]. The fatty acid profile of LDL was measured using the same method as discussed previously for plasma.

2.5. **Cell preparation**

U937 cells (ATCC, Rockville, MD) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 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% CO2-humidified incubator. Hydroxynonenal and HHE (Cayman Chemical, Ann Arbor, MI) were dissolved in ethanol. Cells (2.5~5 x 105/ml) were incubated with 300 µl oxLDL (providing 0.15 mg LDL

cholesterol) obtained from the subjects supplemented with SO, LFO and HFO, 10 μM of HHE, 7 μM of HHE plus 3 μM of HNE, 5 μM of both HHE and HNE, and 10 μM of HNE.

2.6. **Measurement of apoptosis**

Apoptosis was measured by three different methods. The Annexin V fluorescein isothiocyanate (FITC) binding assay was used to measure the translocation of phosphatidylserine from the inner to the outer plasma membrane of the U937 cells by flow cytometry. Briefly, 2.5 x 105 cells were incubated with 300 μl oxLDL, providing 0.15 mg LDL cholesterol, or the appropriate concentrations of the aldehydes for 19 h or 6 h in 12-well plates. After incubation, the cell suspension was removed from each well and washed with ice-cold phosphate-buffered saline solution (PBS). The resulting cell pellet was resuspended in binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2), 4 μl of Annexin V–FITC (PharMingen, San Diego, CA) and then incubated in the dark for 20 min. Cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson) using the FL1 channel to detect the signal generated by FITC. Apoptotic cells were reported as the percentage of 104 cells stained with Annexin V – FITC.

DNA fragmentation of U937 cells was determined by measuring hypodiploid DNA by flow cytometry. Hypodiploid nuclei with a lower DNA content stain less than G1 cells. Their appearance is a marker of apoptotic cell death [37]. Briefly, 3 x 105 U937 cells were incubated with 300 μl of oxLDL, providing 0.15 mg LDL cholesterol, or appropriate concentrations of the aldehydes for 19 or for 6 h in 12-well plates. After incubation, the cell suspension was removed from each well and washed with ice-cold PBS. While vortexing, ice-cold 70% ethanol was added to the cell pellet. The samples were then incubated in the freezer for 30 min. After incubation, the cells were washed with PBS. Binding buffer, RNAase (2 μg/ml, Sigma) and propidium iodide (PI) (5 μg/ml, Sigma) were added to the cell pellets. Samples were held at 4°C for 30 min in the dark. Cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson) using the FL2 channel to detect the signal generated by PI. Analysis of the DNA cell cycle was performed by the CellQuest analysis program (Becton Dickinson). The degree of apoptosis was measured the percentage of hypodiploid nuclei in 104 cells.

In addition to the above two methods, apoptosis was measured in U937 cells after incubation with appropriate concentrations of the aldehydes by condensed chromatin and nuclear fragmentation after staining the cells with the fluorescent dye, 4,6-diamidino-2-phenylindole (DAPI). Briefly, 2.5 x 105 cells were incubated for 19 h with 10 μM of HHE, 7 μM of HHE plus 3 μM of HNE, 5 μM of both HHE and HNE, or 10 μM of HNE. Cells were harvested washed with PBS (pH 7.4, 10 mmol/L) before being resuspended in PBS and loaded on polylysine slides (Sigma) in Cytospin Chambers (StatSpin, Norwood, MA). Slides were cytospun in a Cytofuge (StatSpin) for 4 min at 850 rpm (40xg). After spinning, slides were immersed in 3.7% fresh 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 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 nuclei, characteristic of either condensed or fragmented chromatin, while normal cells were characterized by a faint blue nuclei. About 100 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.

2.7. **Statistical analysis**

Summary statistics were calculated (mean and standard error of the mean). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer test to determine significant differences among treatments. Analyses were completed using the SPSS (SPSS10.1, Chicago, IL). A value of $P<.05$ was considered to be statistically significant.

Table 1
Concentration of selected fatty acids in LDL obtained from postmenopausal women after supplementation with SO, a low dose of fish oil (LFO), or a high dose of fish oil (HFO) for 5 weeks[1]

	SO	LFO	HFO
Relative wt.%			
C14:0	1.08 ± 0.07	1.38 ± 0.10	1.28 ± 0.07
C16:0	20.72 ± 0.58	22.51 ± 0.63	22.84 ± 0.53
C18:0	5.19 ± 0.14	5.12 ± 0.15	5.38 ± 0.16
Σ SFA[2]	28.12 ± 0.66[a]	30.33 ± 0.65[b]	30.83 ± 0.40[b]
C16:1n-7	2.07 ± 0.24	3.21 ± 0.46	3.84 ± 0.46
C18:1n-7	5.60 ± 0.51	5.16 ± 0.47	4.75 ± 0.56
C18:1n-9	14.64 ± 0.44	15.59 ± 0.30	15.80 ± 0.58
Σ MUFA[3]	23.68 ± 0.69	25.51 ± 0.74	26.01 ± 0.79
C18:2n-6	37.50 ± 1.01[a]	32.21 ± 1.27[b]	28.79 ± 0.94[b]
C20:4n-6	6.63 ± 0.29[a]	5.46 ± 0.43[b]	5.24 ± 0.27[b]
C20:5n-3	0.50 ± 0.03[a]	1.94 ± 0.17[b]	3.71 ± 0.24[c]
C22:5n-3	0.30 ± 0.04[a]	0.46 ± 0.04[b]	0.66 ± 0.05[c]
C22:6n-3	0.77 ± 0.06[a]	1.62 ± 0.12[b]	2.17 ± 0.18[c]
Σ PUFA[4]	48.19 ± 0.90[a]	44.19 ± 1.31[b]	43.23 ± 0.82[b]

[1] Values are means±S.E.M. Different superscript letters represent significant differences in LDL concentrations after supplementations ($P<.05$).

[2] Σ SFA = C14:0+C16:0+C18:0+C20:0+C22:0+C24:0.

[3] Σ MUFA = C14:1n-5+C16:1n-7+C18:1n-7+C18:1n-9+C20:1n-9+C22:1n-9+C22:1n-11.

[4] Σ PUFA = C16:2n-4+C16:4n-1+C18:2n-6+C18:3n-3+C18:4n-3+C20:2n-6+C20:3n-6+C20:4n-6+C20:5n-3+C22:5n-3+C22:6n3.

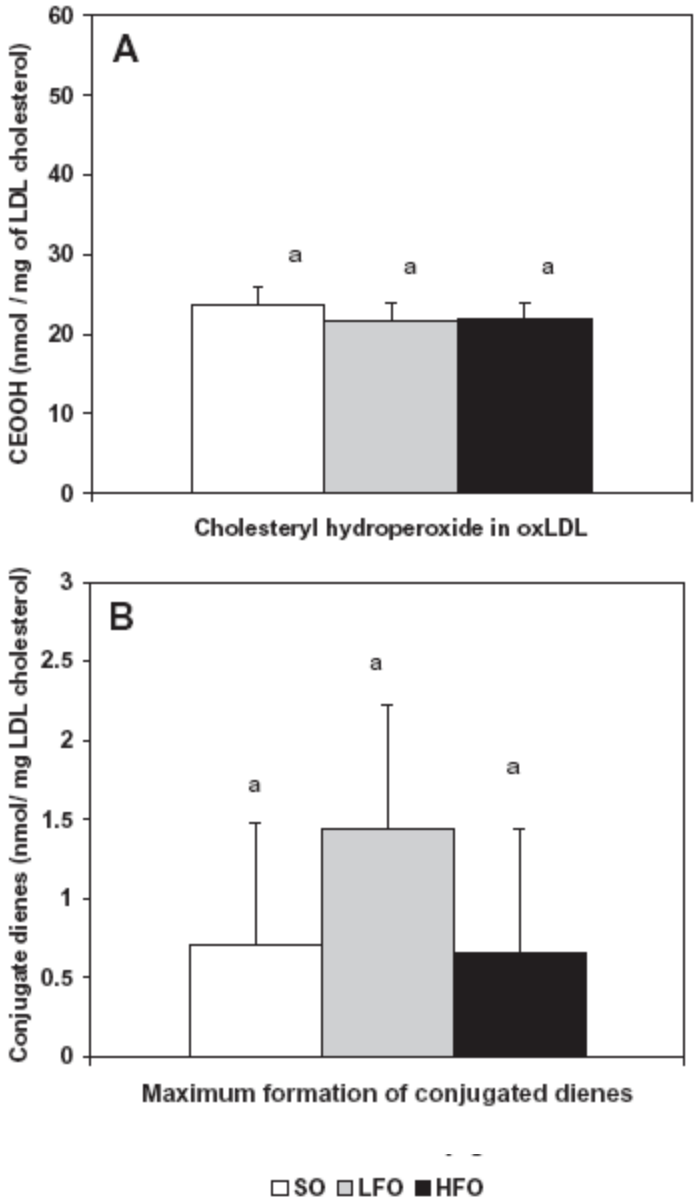


Fig. 1. Cholesteryl ester hydroperoxide (CEOOH) concentration and maximum concentration of conjugated dienes measured in LDL obtained from the subjects supplemented with 14 g/day SO, 7 g/day SO and 7 g/day fish oil (LFO), or 14 g/day fish oil (HFO) and then oxidized with 5 μM $CuSO_4$ at 37°C for 6 h. Data are means±S.E.M. Bars with different letters above them have a significant effect produced by the oil supplementation, $P \leq .05$.

3. *Results*

All of the participants had been receiving HRT for at least 1 year before entering the study. About one third of the participants were on estrogen therapy and two thirds were on estro-progestins. Each participant continued on the same HRT regimen for the duration of the study.

The average age of the subjects was 60±6 years and their average BMI was 25±3 kg/m2. The subjects were normolipemic as indicated by their lipid profile. The average concentration of plasma total cholesterol, LDL-C and HDL-C was 5.57±0.78, 3.29±0.78 and 1.74±0.47 mmol/L (215±30, 127±30 and 67±18 mg/dl), respectively. The average plasma triacylglycerol concentration was 1.25±0.55 mmol/L (111±49 mg/dl).

The intakes of carbohydrate, protein and total fat met the recommendations of the Dietary Guidelines for Americans [38]. The average cholesterol intake was 25% lower than the recommended intake of 300 mg daily. The diets provided 4.4±2.5 mg ot-tocopherol and the supplements supplied an additional 7.8 mg of ot-tocopherol. This made the average intake approximately 12.2 mg/day, which is 19% lower than the recommended intake of 15 mg/day [39].

There was 100% retention of the participants on the study. Compliance to the study was excellent as indicated by the low number of unused capsules returned by the subjects and the change in the plasma fatty acid profile. Plasma EPA and DHA concentrations were significantly increased with fish oil supplementation in a dose-dependent manner. The amount of EPA was significantly higher in the LFO (380%, P=.003)- and the HFO (1000%, P=.000)-supplemented groups compared to the SO-supplemented group, and higher in the HFO (145%, P=.000)-supplemented group compared to the LFO-supplemented group. The amount of DHA was higher in the LFO (98%, P=.002)- and in the HFO (176%, P=.000)-supplemented groups compared to the SO- supplemented group, and higher in the HFO (40%, P=.016)- supplemented group compared to the LFO-supplemented group. Plasma ot-tocopherol concentrations were not different among the groups after supplementation (data not shown).

After supplementation, there were no significant differences seen in the sum of MUFA in LDL as presented in Table 1. However, the sum of the SFA was significantly higher in LFO (7.8%, P=.033)- and HFO (9.6%, P=.008)- supplemented groups compared to the SO-supplemented group. The sum of the PUFA was significantly lower in LFO (8.3%, P=.029)- and HFO (10.2%, P=.006)- supplemented groups compared to the SO-supplemented group. This occurred primarily because the concentration of linoleic acid in the LDL was lower in the LFO (14%, P=.005) and HFO (23%, P=.000) groups compared to the SO-supplemented group. Additionally, the amount of arachidonic acid was lower in the LFO- and HFO- supplemented groups (18%, P=.05 in LFO and 21%, P=.01 in HFO) than the SO-supplemented one. However, the sum of PUFA, linoleic acid and arachidonic acid concentrations were statistically equivalent at both doses of fish oil. In contrast, there was a dose-dependent change in the LDL of the concentration of the (n-3) fatty acids. The amount of EPA was significantly higher in the LFO (288%, P=.000)- and the HFO (642%, P=.000)-supplemented groups compared to the SO-supplemented group. It was also higher in the HFO (91%, P=.000)-supplemented group compared to the LFO-supplemented group. The amount of DHA was higher in the LFO (110%, P=.000)- and in the HFO (280%, P=.000)-supplemented groups compared to the SO-supplemented group and higher in the HFO (34%, P=.016)-supplemented group compared to the LFO-supplemented group.

3. 1. **Oxidized products in LDL obtained from subjects after supplementation**

CEOOH concentration and maximum concentration of conjugated dienes in oxLDL obtained after the oil supplementation are shown in Fig. 1. These two measurements show no differences in oxidized products among the three treatment groups.

3.2. **Apoptosis of U937 cells after incubation with oxLDL**

The percent of apoptotic U937 cells after incubation with oxidized LDL obtained from subjects following supplementation with the three treatment oils is shown in Fig. 2. The percent of apoptotic cells was approximately 40% lower in the HFO-supplemented group than in the SO-supplemented group measured by both the Annexin V and the DNA fragmentation assays (P=.04 and .004, respectively). The percent of apoptotic cells was also lower in the LFOsupplemented group. However, it was statistically equivalent to the percentage measured after both the SO supplementation and the HFO supplementation.

3.3. Apoptosis of U937 cells after incubation with HNE and HHE

Apoptosis of U937 cells measured after incubation with the aldehydes is shown in Fig. 3. The cells were incubated with 10 μM of ethanol (control), 10 μM of HHE, 7 μM of HHE plus 3 μM of HNE, 5 μM of both HHE and HNE, or 10 μM of HNE. Regardless of the method used, the percentage of apoptotic cells was lower in all the control samples than in the samples that contained the hydroxyalkenals. All three concentrations of HHE, regardless of the concentration of HNE, gave a statistically equivalent degree of apoptosis by all three measurements. In addition, it was lower in the systems that contained HHE at any level than in the systems that contained only HNE. Apoptosis of U937 cells was approximately 40% (P=.001) lower in cells incubated with 10 gM of HHE or the mixtures of HHE and HNE than 10 gM HNE when measured by the Annexin V assay. Apoptosis of U937 cells was 51% (P=.004) and 64% (P=.0001) lower in cells incubated with 10 gM of HHE and mixtures of HHE and HNE than 10 gM of HNE by the DNA fragmentation and DAPI staining assays, respectively.

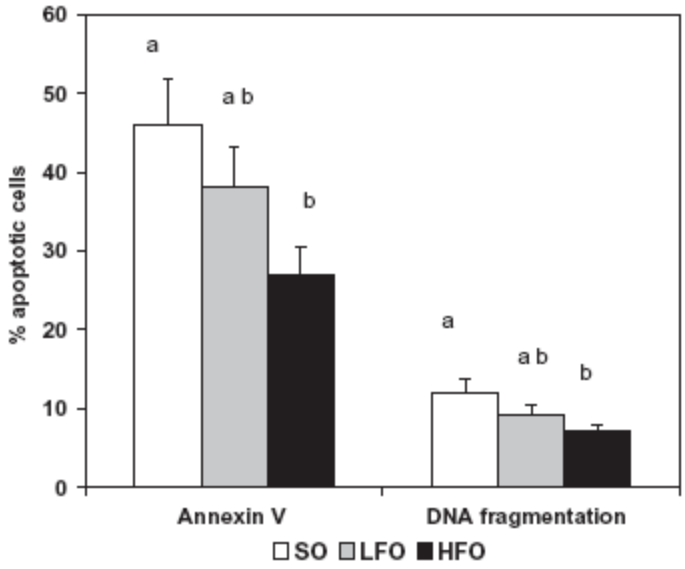
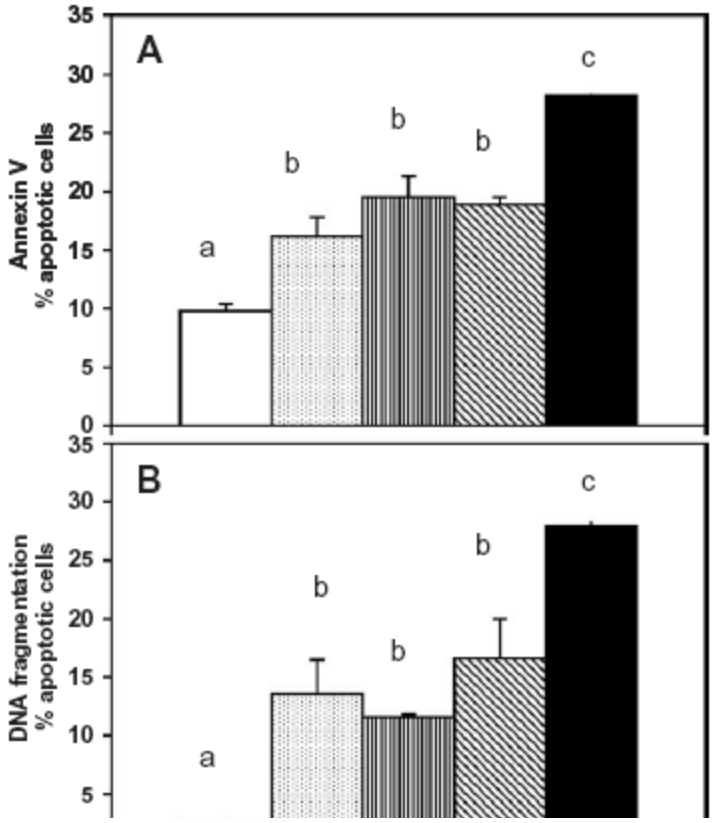


Fig. 2. U937 cells were incubated with oxidized LDL, providing 0.15 mg LDL cholesterol, obtained from the subjects supplemented with 14 g/day SO, 7 g/day SO and 7 g/day fish oil (LFO), or 14 g/day fish oil (HFO). Apoptosis of U937 cells was measured by the Annexin V fluorescein isothiocyanate (FITC) binding assay and DNA fragmentation. Apoptotic cells were reported as the percentage of 10^4 cells stained with Annexin V–FITC or the percentage of hypodiploid nuclei in 10^4 cells. Data are means±S.E.M. Bars with different letters above them have a significant effect produced by the oil supplementation, $P \leq .05$.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Annexin V
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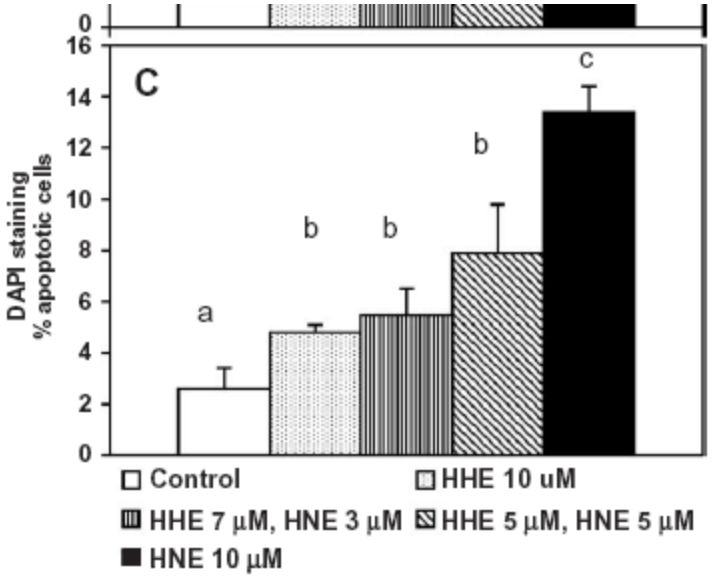

Fig. 3. U937 cells were incubated with 10 μM of ethanol (control), 10 μM of HHE, 7 μM of HHE plus 3 μM of HNE, 5 μM of both HHE and HNE, or 10 μM of HNE. Apoptosis was measured by the Annexin V fluorescein isothiocyanate (FITC) binding assay, DNA fragmentation and DAPI staining. Apoptotic cells were reported as the percentage of 10^4 cells stained with Annexin V–FITC, the percentage of hypodiploid nuclei in 10^4 cells or the percentage of DAPI-stained cells from three randomly selected fields on each slide. Data are means± S.E.M. Bars with different letters above them have a significant effect produced by the oil supplementa-

4. *Discussion*

In the last 15 years, several clinical trials [40–44] have provided compelling evidence that EPA and DHA, consumed either from fish or fish oil capsules, are cardioprotective. In particular, the fish oil fatty acids have been shown to be effective in preventing sudden cardiac death [8,45]. Although it has been suggested that the role of EPA and DHA in prevention of sudden cardiac death is explained by their antiarrhythmic or antithrombotic effects [46], Thies et al. [47] found that the n-3 fatty acids also enhanced the stability of atherosclerotic plaques through their antiinflammatory function and suggested that this could be the basis of their effects on CVD. In addition, the manner in which EPA and DHA modulate apoptosis may impact the readiness with which a plaque ruptures.

Apoptosis clearly has a role in plaque stability, although whether this is a beneficial or detrimental function remains to be determined [16]. Apoptotic vascular cells have been found in atherosclerotic lesions [13]. Since oxLDL has been reported to induce apoptosis of endothelial cells, smooth muscle cells, fibroblasts and monocytes/macrophages in vitro [18], it may be a potential inducer of apoptosis of vascular cells in vivo. The oxidized lipids in oxLDL are thought to be responsible for the apoptosis that it induces. Thus, events that modify the bioactive lipid profile of oxLDL may impact its ability to induce apoptosis, and ultimately, plaque stability.

We previously reported that oxLDL enriched with EPA and DHA markedly lowered the extent of apoptosis in the promonocytic U937 cell line [24]. In the current study, a much larger

supplementation trial, we confirmed that EPA/ DHA-enriched oxLDL induced less apoptosis in U937 cells compared to oxLDL enriched with linoleic acid. In addition, HHE either singly or when mixed with HNE produced fewer apoptotic cells than HNE alone. These observations were verified by using several standard apoptotic markers. A limitation to this study is the use of U937 cells. These cells are an undifferentiated promonocytic cell line used to represent a model for the macrophages found in the atherosclerotic lesion. A more complete picture would be obtained by performing similar experiments with human monocytes and/ or macrophages taken from atherosclerotic lesions.

When differences are produced in physiologic systems by the long chain n-3 fatty acids compared to the n-6 fatty acids, the usual argument is that the n-3 fatty acids cause increased oxidative stress. However, we have shown [48] that in postmenopausal women supplemented with fish oil at an amount similar to that which was used in the current study, there was no increase in in vivo oxidation. We also showed in this study as well as in an earlier pilot study [24] that supplementation with fish oil did not increase the concentration of cholesteryl ester hydroperoxides or conjugated dienes compared to supplementation with oils that contained fewer highly unsaturated fatty acids. Further, we suggested [49] that the array of oxidation products produced by the n-3 fatty acids differed from the array produced by the n-6 fatty acids and that this difference produced changes in physiologic responses. Thus, the purpose of the current study was to test this hypothesis by determining if the hydroxyaldehydes, HNE and HHE, derived from oxidation of n-6 and n-3 fatty acids, respectively, could contribute to the difference in apoptosis produced by oxLDL.

In both studies, modest amounts of EPA and DHA were consumed in the form of fish oil. The amounts used were similar to the American Heart Association's recommended intakes of 1 g/day of EPA plus DHA for patients with existing CVD [50] and have been used in secondary prevention trials [42]. The 7 g of fish oil given to the LFO group were equivalent to what could be obtained from one serving (100 g) of fatty fish and the 14 g could be obtained from two servings. The 14 g of SO contained 9.8 g of linoleic acid. This amount could be obtained from approximately three teaspoons of corn oil.

In addition to using oil supplementation at levels that were physiologically relevant, the amount of HNE and HHE to which the U937 cells were exposed was also physiologically appropriate. Hydroxynonenal is a normal constituent of plasma and various types of cells. Steady-state concentrations range from 0.2 to 2.8 μM [27]. Although the concentration of HNE in the oxLDL was not measured in this study, Muller et al. [22] reported HNE concentrations of 22–114 nmol/mg LDL protein and HHE concentrations of 4–49 nmol/mg LDL protein in copper-oxidized LDL. The concentration of each aldehyde increased as the extent of oxidation increased. From these data, we can estimate that the concentration of HNE presented by the EPA/DHAenriched oxLDL to the U937 cells in this study ranged from approximately 2–10 μM. Extrapolation from Mqller's data for the HHE concentrations would yield values that are too low because their LDL was not EPA/DHA enriched. However, these calculations may represent a minimum level of HNE and HHE that can participate in in vivo processes. Since these aldehydes remain largely associated with the LDL particle [51], Esterbauer and Ramos [52] estimated that the concentration of HNE in the lipid phase of strongly oxidized LDL could be as high as 150 mM.

A less reactive role of HHE compared to HNE is not without precedent. Hydroxynonenal and HHE are lipophilic, relatively diffusible and highly reactive. Each contains a carbonyl group, a C2/C3 double bond and a C4-hydroxyl group. Hydroxynonenal contains nine carbons in its aliphatic chain, while HHE contains only six. Poli and Schaur [53] showed increased steady-state levels of the aldehydes in a variety of pathological disease processes occurring in humans, including atherosclerosis, respiratory distress syndrome and neuronal degenerative disorders. They suggested [53] that the role of the aldehydes during pathophysiological conditions could be due to selective alteration in cell signaling, protein or DNA damage, cell proliferation and cell differentiation, and induction of apoptosis. Muller et al. [22] investigated the cytotoxic and chemotactic potencies of several aldehydes, including HNE and HHE. They found that HNE was more toxic than HHE. The increased toxicity may be because of the greater lipophilicity of the longer aliphatic chain [54].

The physiologic significance of fish oil consumption on plaque stability through the impact of apoptosis of macrophages at this point is mere supposition. On the one hand, it could decrease plaque stability. Kockx and Herman [16] suggested that loss of macrophages in plaques would lead to less metalloproteinase activity and to decreased collagen breakdown. This could lead to plaque stabilization, and therefore, decrease the risk for plaque rupture. Thus, EPA and DHA would result in more macrophages, more collagen breakdown and decreased plaque stability. On the other hand, fish oil consumption may be associated with increased plaque stability. Macrophages are responsible for scavenging apoptotic bodies. If apoptotic bodies accumulate, complement and thrombin activation could increase [16], resulting in plaque rupture. Less apoptosis of macrophages would suggest increase removable of apoptotic bodies.

This study suggests that the type of oxidized bioactive lipid found in oxLDL may offer another largely unexplored role of the n-3 fatty acids on apoptosis, and ultimately, plaque stability. Knowledge of this nature is necessary to make the most effective use of EPA and DHA to alter the course of CVD.

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