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Original Article

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***Aim*: Dietary supplements in polyunsaturated fatty acids (PUFA), particularly omega-3, are well known for their beneficial effects in preventing cardiovascular diseases (CVD). The aim of this study was to determine the role of PUFA on the modulation of apoptosis induced by hypochlorous acid-oxidized LDL (HOCl-oxLDL) in U937 cells.**

***Methods*: We tested the effect of monocyte cell line U937 supplementation with eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (ARA) or oleic acid (OA) on the modulation of HOCl-oxLDL-induced apoptosis.**

***Results*: First, we showed the incorporation of fatty acids in the cellular membrane in U937 cells. Then, we showed that both EPA and ARA exerted a pro-apoptotic effect through the intrinsic mitochondrial apoptotic pathway including the dissipation of mitochondrial membrane potential followed by cardiolipin depletion, the downstream activation of caspase-3 and the increase in DNA fragmentation. The pro-apoptotic effect of EPA or ARA was completely blocked in U937/Bcl-2 cells.**

***Conclusions*: A new mechanism of dietary supplements in PUFA with likely consequences in apoptosis could be suggested through the mitochondrial pathway in monocytes.**

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Key words; Fatty acids, Lipoproteins, Apoptosis, Monocytes, Atherosclerosis

Introduction

Atherosclerosis is a chronic inflammatory disease of the vascular wall and represents the major cause of cardiovascular events[1)]. The pathology is initiated by the adhesion of circulating monocytes to the endothelium and their subsequent transmigration into the subendothelial space where they differentiate into macrophages and acquire the ability to take up oxidatively modified low density lipoproteins (oxLDL) and become foam cells[2-4)]. Intracellular accumulation of cholesterol-loaded macrophages in atherosclerotic lesions is a prominent feature throughout the life of the lesion, and these cells have a tremendous impact on lesion progression[5-7)].

Apoptosis is a selective, controlled, and genetically programmed cell death process that plays an important role in the balance between cell replication and cell death and has been recognized to play a part in a number of vascular diseases[8)]. The intrinsic mitochondrial apoptotic pathway was shown to be mainly activated by oxLDL, at least in monocytes[9)]. In a recent review focused on the consequences and therapeutic

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implications of monocyte-macrophage apoptosis in atherosclerosis, Tabas[10] discussed the effect of cell death on plaque progression depending on the lesion stage during which it occurs. Moreover, the author underlined the importance of the efficiency of apoptotic cell clearance by intimal phagocytes, added to a decreased number of pro-atherogenic macrophages, leading to an overall anti-atherogenic effect in early lesions. In contrast, the author suggested that clearance of apoptotic macrophages in advanced lesions is defective because of necrosis, inflammation and thrombosis. Indeed, in addition to apoptosis in advanced atherosclerotic plaques, macrophages and lipid-loaded foam cells undergo necrosis, thereby releasing their intracellular content due to cytolysis resulting in the formation of the lipid-rich core of atheromatous plaque.

Dietary intake of omega-3, very long chain PUFA found in fish, especially EPA and DHA, and consumption of seafood (1-2 servings per week) are associated with decreased CVD mortality in humans[11, 12]. Clinical studies have demonstrated that the stability of atherosclerotic plaques could explain the reduction in non-fatal and fatal cardiovascular events associated with increased n-3 PUFA intake[13]. These effects might involve changes in triglyceride blood levels and lipoproteins leading to a less atherogenic profile by slowing down the progression of atherosclerosis[14]. Recently, it was demonstrated that fish oil reduced the expression of P-selectin and vascular and intracellular adhesion molecule (VCAM-1 and ICAM-1), which may explain the prevention of atherosclerosis development[15].

Thus, we hypothesized a beneficial role of PUFA through the modulation of the oxLDL-induced apoptosis of monocytes at the vascular level. The aim of this study was to investigate the role of EPA (20:5 n-3) and DHA (22:6 n-3), compared to ARA (20:4 n-6) and OA (18:1 n-9) in U937 cell apoptosis. Through their pro-apoptotic effect observed *in vitro*, EPA and ARA could inhibit monocyte infiltration and macrophage accumulation in arterial intima and thus, by slowing down the progression of atherosclerosis, might be considered as nutritional protectors in the development of CVD.

Materials and Methods

Cell culture

The human leukemic monocyte lymphoma cell line U937 and U937/Bcl-2 cells were cultured as previously described[9]. To investigate the effect of fatty acid (FA) supplementation, cells were pre-incubated for 24 h with an FA-enriched culture medium containing a final molar ratio FA/BSA close to 2, corresponding to 80 μM of added FA. EPA, DHA, ARA or OA (Cayman Chemical Company) linked to bovine serum albumin (BSA), for 3 h at 37℃, by mixing free FA/BSA/FCS (fetal calf serum) and adding to the culture medium. Only BSA was added to the control culture medium. Experiments were performed with cells cultured at a concentration of 1.10^6 cells/mL. Cells grew in FA-enriched culture medium for 24 h, and then native LDL or oxLDL was added to the medium at the indicated concentration (in term of LDL proteins) and for the indicated time to induce apoptosis.

LDL isolation and modification

Lipoproteins were separated from human plasma of healthy blood donors by sequential ultracentrifugation and the LDL fraction was isolated as previously described[16]. LDL oxidation was induced for 30 min at 37℃ with 4 mmol/L HOCl (corresponding to an oxidant/protein molar ratio of 2000/1). Untreated and oxidized LDL were dialyzed overnight against isotonic PBS. The LDL protein concentration was determined using the bicinchoninic acid (BCA) assay (Sigma).

Fatty acid analysis

The cells were collected in 1 mL distilled water and lipids were extracted in a vol/vol chloroform-methanol mixture according to Bligh and Dyer[17]. FA were transmethylated with BF3-methanol and then the methyl esters were analyzed by gas chromatography on an econocap EC-WAX capillary column (0.32 x 30 m; Alltech Associates) using heptadecanoic acid (C17:0) as the internal standard. Using this method, it was demonstrated that the major percentage of FA is included in the cell membrane[18]. Since the phospholipids represent in these cells more than 95% of total lipids, the FA composition determined in this fraction was considered as representative of the membrane phospholipid FA composition.

Apoptosis assays

DNA fragmentation

Apoptotic cell death was quantified using cell cycle analysis by flow cytometric quantification of DNA content using a fluorescence-activated cell sorter (FACS). Following treatment, cells were collected, washed twice with PBS, resuspended in ice-cold 70% ethanol with gentle vortexing, and stored at −20℃ until analysis. For DNA quantification, cells were resuspended into 200 μL appropriate buffer containing 50 μg/mL propidium iodide (PI) (Sigma). DNA content analysis of 10,000 cells was conducted using a FACScan flow cytometer (Becton Dickinson). Cells

with a normal DNA content (at least diploid 2 N) were scored as viable, whereas cells with a hyplodiploid DNA content (lower than 2 N) were scored as apoptotic.

Caspase-3 activity

After treatment, the cells were collected and protein concentrations were evaluated using the BCA assay and then caspase-3 activity was determined from 100 μg total protein extracts in 100 μL reaction mixture using the Ac-DEVD-AFC N-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl coumarin) synthetic fluorogenic substrate (Biomol) in the following buffer (20 mM PIPES pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 10 mM DDT). The release of AFC from the substrate was measured after 2 h as emission at 505 nm upon excitation at 400 nm using a microplate fluorescence reader (TECAN).

Western blot analysis

Pro-caspase-3 (35 kDa) and activated cleaved fragments of caspase-3 (17 kDa and 19 kDa) were determined using 50 μg protein extracts. After treatment, cells were collected, protein concentrations were evaluated using the BCA assay and then separated by Western blotting on NuPAGE 4-12% Bis-Tris gels (Invitrogen). After electrophoretic transfer to nitrocellulose membranes and blockade, the membranes were incubated with primary antibodies. Caspase-3 was detected with rabbit anti-human monoclonal antibody (Cell Signalling). Anti-rabbit IgG conjugated to horseradish peroxidase was used as the secondary antibody. Immunoreactive bands were detected with the enhanced chemiluminescence system ECL (Amersham Biosciences).

Mitochondrial membrane potential ($\Delta\Psi_m$)

The inner membrane of mitochondria is characterized by a transmembrane potential $\Delta\Psi_m$ generated through the activity of a proton pump in the respiratory chain. The cells were loaded with a mitotracker fluorochrome (3,3'-dihexyloxacarbocyanine iodide, $DiOC_6$) (Molecular Probes, Inc.) at 40 nM final concentration for 30 min. The dye accumulates in mitochondria when membrane potential is intact and the fluorescence of $DiOC_6$ can therefore be considered as an indicator of the relative mitochondrial membrane polarization state. Relative fluorescence intensities from 10,000 cells were measured on a FACScan flow cytometer (Becton Dickinson).

Measurement of mitochondrial cardiolipin depletion

After treatment, the cells were collected in 1 mL distilled water and lipids were extracted in a vol/vol chloroform-methanol mixture according to Bligh and Dyer[17]. Lipids were separated by high performance liquid chromatography (HPLC) (Agilent Technologies) on a PVA-Sil column (PVA-Sil 120A, S-5 μm) at 35℃ and the peak corresponding to cardiolipin was quantified by a light scattering detector (Eurosep).

Statistical Analysis

The data are expressed as the means ± SEM, and were submitted to one-way or two-way analysis of variance (ANOVA). When statistically different, the means were compared by the Newman-Keuls test[19].

Results

Fatty acid incorporation into cells

Table 1 shows the incorporation of FA added to the culture medium during 24 h into U937 cells. EPA (20:5 n-3), DHA (22:6 n-3), ARA (20:4 n-6) or OA (18:1 n-9) showed a 19-, 7-, 5- or 2-fold increase, respectively. The membrane composition was characterized by a significant decrease in saturated FA (approximately -25% vs control cells) in EPA-, ARA- and OA-enriched cells but not in DHA- enriched cells. Similarly, the MUFA content was significantly decreased and the PUFA content significantly increased in PUFA- supplemented cells, but not in OA- enriched cells. Supplementation with EPA induced a 19-fold increase in EPA content and a 8-fold increase in DPA (docosapentaenoic acid, 22:5 n-3). Supplementation with either n-3 PUFA (EPA or DHA) induced a significant decrease in ARA content by comparison with the control (44% and 35%, respectively). Supplementation with ARA induced a net increase in n-6 PUFA and a slight decrease in n-3 PUFA. All supplementations with PUFA resulted in an increased p/s ratio but supplementation with OA did not.

Effects of fatty acids on DNA fragmentation

We first investigated the time and dose required to induce cell apoptosis as assessed by DNA fragmentation (sub-G1 peak) in native LDL- or HOCl-ox-LDL- treated cells (data not shown). These experiments identified 24 h exposure at 250 μg/mL as the most efficient condition. **Fig. 1** shows a significant increase in the proportion of cells undergoing spontaneous apoptosis, as observed in the presence of native LDL treatment in EPA- or ARA-enriched cells (approximately 5%) as compared to control cells (2.5% ± 0.25). By contrast, no significant modification was

Table 1. Percentage of fatty acids in U937 membranes after 24 h in a medium enriched with EPA, DHA, ARA or OA

	control	EPA	DHA	ARA	OA
14:00	1.8 ± 0.40	1.2 ± 0.16	1.6 ± 0.22	1.5 ± 0.28	1.3 ± 0.13
15:00	0.5 ± 0.13	0.4 ± 0.09	0.5 ± 0.08	0.5 ± 0.10	0.5 ± 0.05
16:00	20.8 ± 0.83	15.5 ± 1.03	20.3 ± 0.61	17.1 ± 1.09	15.3 ± 0.78
Σ16:1	7.5 ± 0.69	3.2 ± 0.25	4.5 ± 0.20	3.5 ± 0.22	3.6 ± 0.14
18:00	16.6 ± 1.30	11.4 ± 0.56	14.5 ± 0.93	11.8 ± 0.74	13.0 ± 0.90
18:1n-9	18.8 ± 0.30	11.3 ± 0.84	13.6 ± 0.71	11.4 ± 0.82	39.5 ± 1.92
18:1n-7	8.5 ± 0.84	3.9 ± 0.05	4.9 ± 0.25	4.3 ± 0.14	5.7 ± 0.52
18:2n-6	4.5 ± 1.44	3.9 ± 1.17	5.1 ± 1.27	3.7 ± 1.13	4.3 ± 1.28
Σ18:3	1.0 ± 0.35	1.0 ± 0.22	1.1 ± 0.24	1.0 ± 0.27	0.8 ± 0.25
20:00	0.4 ± 0.15	0.2 ± 0.08	0.3 ± 0.12	0.1 ± 0.06	0.3 ± 0.13
Σ20:1	0.7 ± 0.14	0.2 ± 0.05	0.3 ± 0.07	0.2 ± 0.08	1.6 ± 0.46
20:2n-6	1.0 ± 0.24	0.3 ± 0.07	0.3 ± 0.06	0.3 ± 0.11	0.2 ± 0.11
20:3n-6	1.6 ± 0.06	1.1 ± 0.10	1.2 ± 0.09	1.8 ± 0.29	1.1 ± 0.10
20:4n-6	5.2 ± 0.39	2.9 ± 0.34	3.5 ± 0.38	27.0 ± 3.97	3.7 ± 0.25
20:5n-3	1.1 ± 0.07	20.6 ± 3.4	2.1 ± 0.39	0.5 ± 0.11	0.7 ± 0.12
22:00	0.5 ± 0.09	1.0 ± 0.32	0.4 ± 0.05	0.3 ± 0.05	0.3 ± 0.04
22:2n-6	1.6 ± 0.33	1.2 ± 0.29	1.2 ± 0.30	1.2 ± 0.37	1.3 ± 0.09
22:4n-6	0.4 ± 0.13	0.3 ± 0.11	0.2 ± 0.06	8.2 ± 2.57	0.6 ± 0.11
22:5n-6	0.1 ± 0.04	0.1 ± 0.04	0.1 ± 0.05	0.4 ± 0.27	0.1 ± 0.07
22:5n-3	2.1 ± 0.07	16.2 ± 2.28	1.9 ± 0.14	1.7 ± 0.09	1.8 ± 0.15
24:00	0.9 ± 0.19	0.4 ± 0.07	0.6 ± 0.06	0.4 ± 0.06	0.5 ± 0.09
22:6n-3	2.8 ± 0.37	2.1 ± 0.13	20.3 ± 2.51	2.0 ± 0.28	2.0 ± 0.11
24:1n-9	1.3 ± 0.26	0.7 ± 0.18	0.9 ± 0.19	0.6 ± 0.17	1.7 ± 0.20
Σ SFA	41.6 ± 0.50	30.1 ± 1.71	38.4 ± 0.93	31.9 ± 1.21	31.3 ± 0.95
Σ MUFA	36.2 ± 1.73	19.2 ± 1.30	24.0 ± 0.78	20.0 ± 1.27	51.1 ± 2.13
Σ PUFA	21.4 ± 2.01	50.3 ± 2.85	37.3 ± 0.69	47.9 ± 2.20	16.5 ± 1.73
Σ PUFA n-6	14.4 ± 1.50	9.8 ± 1.75	11.7 ± 1.87	42.9 ± 2.51	11.3 ± 1.31
Σ PUFA n-3	7.0 ± 0.60	40.5 ± 4.36	25.5 ± 2.18	5.0 ± 0.55	5.2 ± 0.45
n-6/n-3	2.1 ± 0.11	0.3 ± 0.09	0.5 ± 0.12	9.3 ± 1.31	2.1 ± 0.11
p/s	0.5 ± 0.05	1.7 ± 0.20	1.0 ± 0.04	1.5 ± 0.13	0.5 ± 0.05
UI	93.7 ± 2.72	237.3 ± 18.16	188.3 ± 10.64	97.1 ± 10.86	97.0 ± 1.63

SFA: Saturated Fatty Acid; MUFA: Monounsaturated Fatty Acid; PUFA: Polyunsaturated Fatty Acid; EPA: Eicosapentaenoic Acid; DHA: Docosahexaenoic Acid; ARA: Arachidonic Acid; OA: Oleic Acid; UI: Unsaturation Index; p/s: PUFA/SFA ratio (FA < 0.1% were omitted). Mean ± SEM, $n = 4$ independent experiments

observed after DHA or OA enrichment. These results suggest a specific pro-apoptotic effect of 20-carbon PUFA under basal conditions devoid of apoptotic stimulus. Whatever the FA modification, HOCl-oxLDL induced a significant increase in DNA fragmentation. Under these stimulating conditions, EPA and ARA (and EPA significantly more than ARA) also elicited significantly higher DNA fragmentation than the 3 other groups (control, DHA and OA).

Effects of fatty acids on the activation of caspase-3

Treatment of the cells with HOCl-oxLDL (250 μg/mL-16 h) resulted in a significant increase in caspase-3 activity, whatever the FA added (**Fig. 2A**); however, the activity of caspase 3 was strongly influenced by FA supplementation, as evidenced by the statistically significant cross-interaction between the FA effect and the treatment effect (HOCl-oxLDL). Under basal conditions (native LDL) EPA- and ARA- enriched cells displayed an enhanced caspase-3 activity, as compared with the 3 other groups (control, DHA and OA). After HOCl-oxLDL treatment, caspase-3 activity was highest in the same two groups (EPA and ARA). Additionally, the activity was significantly in-

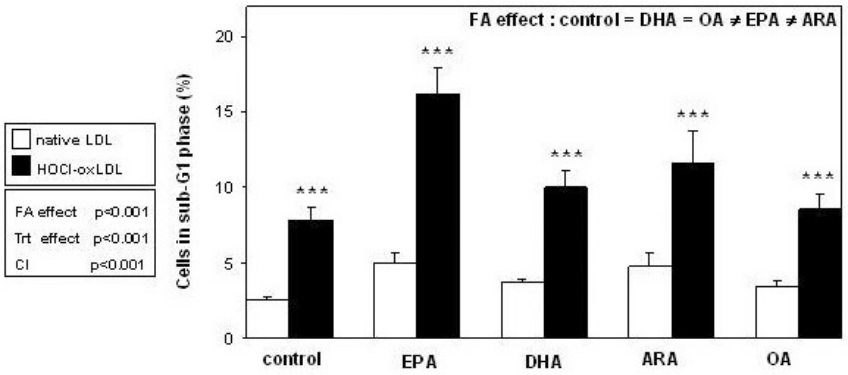

Fig. 1. Effect of EPA, DHA, ARA or OA supplementation on DNA fragmentation after treatment with LDL (250 μg/mL-24 h).

Apoptosis was assessed by DNA content analysis using FACS. Cells undergoing apoptosis with fractional DNA content are presented as the percentage of total events collected. Mean ± SEM, $n=4$ independent experiments. Results of two-way ANOVA are indicated in the inserted table, the effect of FA is explained at the top of the graph and asterisks correspond to the difference between native LDL and HOCl-ox LDL (***: $p<0.0001$).

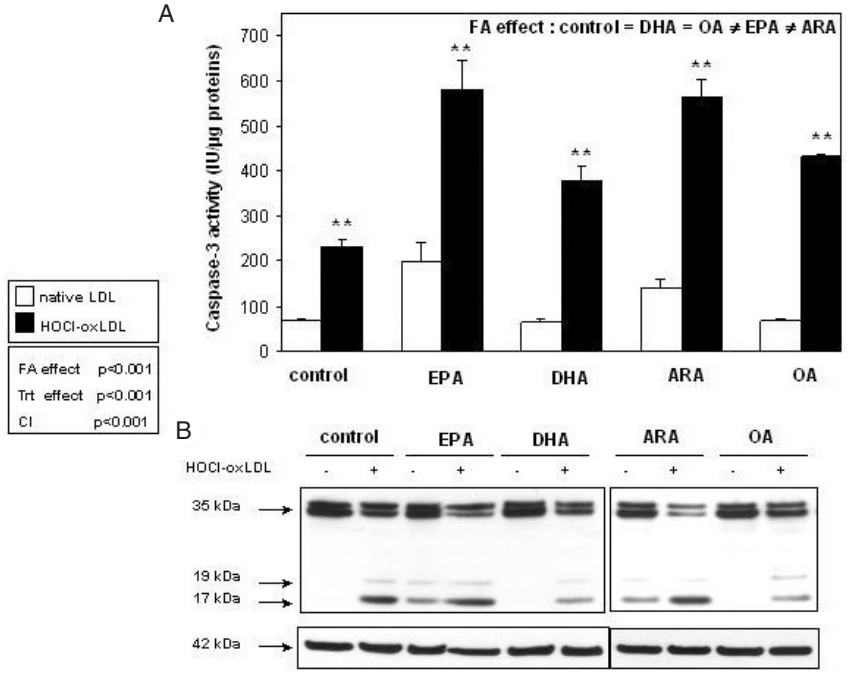

Fig. 2. Effect of FA on caspase-3 activity and expression of activated cleaved fragments.

Cells were treated with EPA, DHA, ARA or OA for 24 h and caspase-3 activity was measured after treatment with LDL (250 μg/mL-16 h). A) Caspase-3 activity was analyzed in cell lysates and expressed as fluorescence values normalized to protein content. Mean ± SEM, $n=3$ independent experiments. Results of two-way ANOVA are indicated in the inserted table, the effect of FA is explained at the top of the graph and asterisks correspond to the difference between native LDL and HOCl-ox LDL (**: $p<0.01$). B) Western blot analyses of pro-caspase-3 (35 kDa) and activated cleaved fragments of caspase-3 (17 kDa and 19 kDa) from cell protein extracts after treatments. β-actin (42 kDa) was used as a protein loading control and did not show any modification. Experiments were repeated at least three times and a representative result is shown.

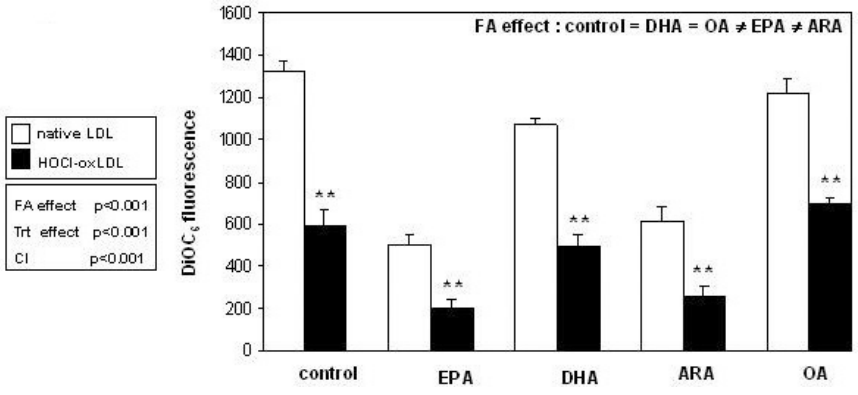

Fig. 3. Effect of EPA, DHA, ARA or OA supplementation on the modulation of $\Delta\Psi_m$ after treatment with LDL (250 μg/mL-16 h).

Cells were collected and labeled with $DiOC_6$, and $\Delta\Psi_m$ was analyzed by FACS. Cells with normal $\Delta\Psi_m$ exhibited strong $DiOC_6$ fluorescence and cells with low fluorescence reflected loss of $\Delta\Psi_m$. Mean ± SEM, $n=4$ independent experiments. Results of two-way ANOVA are indicated in the inserted table, the effect of FA is explained at the top of the graph and asterisks correspond to the difference between native LDL and HOCl-ox LDL. (**: $p<0.01$).

creased in DHA- and OA- enriched cells by comparison with the control, although the basal activity was similar in these 3 groups. Thus, supplementation with EPA and ARA affected both the basal activity and amplitude of the response to HOCl-oxLDL, whereas supplementation with DHA and OA affected only the activation of caspase 3 by HOCl-oxLDL treatment. In **Fig. 2B**, we observed by Western blot analyses the activated form of caspase-3 after EPA or ARA treatment of cells in the presence of native LDL. Furthermore, HOCl-oxLDL treatment (250 μg/mL-16 h) accentuated the activation of caspase-3 and the corresponding release of cleaved fragments. Thus, spontaneous apoptosis appeared after EPA or ARA enrichment and not after DHA or OA treatments. We demonstrated here the synergic activation of caspase-3 after HOCl-oxLDL treatment (250 μg/mL-16 h) reflecting the pro-apoptotic effect of these two FA.

Effects of fatty acids on the $\Delta\Psi_m$

As mitochondrial dysfunction occurs earlier than DNA fragmentation, we investigated the time and dose required to induce cell apoptosis measured by DiOC6 fluorescence in native LDL- or HOCl-oxLDL-treated cells (data not shown). These experiments identified 16 h exposure at 250 μg/mL as the most efficient condition. As shown in **Fig. 3**, enrichment with EPA or ARA was accompanied by a significant decrease in fluorescence reflecting a net decrease in $\Delta\Psi_m$ under basal (unstimulated) conditions. By contrast, treatment with DHA or OA did not influence $\Delta\Psi_m$ under basal conditions. Whatever the FA modification, HOCl-oxLDL induced a significant decrease in $\Delta\Psi_m$ the amplitude of which was weakly affected by FA modifications. Thus, HOCl-oxLDL induced in EPA or ARA cells a further decrease in $\Delta\Psi_m$, which resulted is an extremely low $\Delta\Psi_m$, -85% for both EPA or ARA vs normal culture conditions.

Effects of fatty acids on the cardiolipin level

Cardiolipin is a diphospholipid required for the structural integrity of mitochondria and for the proper function of the electron transport chain. The effects of individual (n-3) and (n-6) PUFA supplementation on total steady-state cardiolipin levels were investigated and the results are shown in **Fig. 4**. These data demonstrated a significant decrease of the cardiolipin level in EPA or ARA cells as compared to control cells under basal conditions, but not in DHA or OA cells. Moreover, under each condition, oxLDL treatment induced significant cardiolipin depletion in cells.

Effects of fatty acids on DNA fragmentation in U937/Bcl-2 cell model

To gain mechanistic insights into the pro-apoptotic effect of EPA or ARA and underlying the involvement of the apoptotic pathway, we performed experiments using the U937/Blc-2 cell model. **Table 2** shows that in this model the proportion of cells undergoing spontaneous apoptosis was similar regardless of the FA added either in the absence or presence of HOCL-ox LDL.

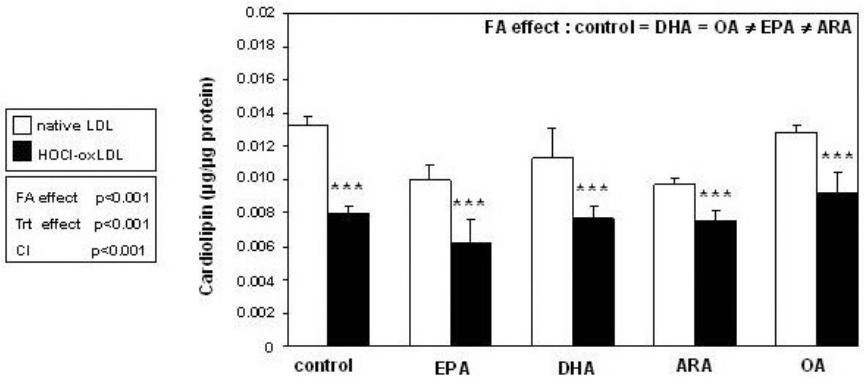

Fig. 4. Effects of FA on cardiolipin concentration.

Lipids were extracted from U397 treated with FA (24 h) and then LDL (250 μg/mL-16 h). Cardiolipin was analyzed using high performance chromatography (HPLC) as described in "Materials and methods" section. Levels of cardiolipin are expressed as μg/μg protein. Mean ± SEM, $n=4$ independent experiments. Results of two-way ANOVA are indicated in the inserted table, the effect of FA is explained at the top of the graph and asterisks correspond to the difference between native LDL and HOCl-ox LDL (***: $p<0.0001$).

Table 2. Effect of FA on DNA fragmentation in U937/Bcl-2 cells after treatment with LDL (250 μg/mL-24 h)

Treatment	control	EPA	DHA	ARA	OA
native LDL	1.51 ± 0.11%	1.48 ± 0.04%	1.42 ± 0.04%	1.63 ± 0.12%	1.32 ± 0.06%
HOCl-ox LDL	1.60 ± 0.16%	1.50 ± 0.08%	1.49 ± 0.10%	1.54 ± 0.06%	1.56 ± 0.10%

Apoptosis was assessed by DNA content analysis using flow cytometry. Cells in sub-G1 phase and undergoing apoptosis are presented as a percentage of the total events collected.
Mean ± SEM, $n=3$ independent experiments. ANOVA results indicated no significant difference between FA supplementation and treatment with LDL (HOCL-ox LDL vs native LDL).

Discussion

This study investigated the impact on U937 monocyte apoptosis of membrane FA composition changes due to incubation in EPA-, DHA-, ARA- or OA-supplemented medium. Here, we tried to correlate the beneficial effect of PUFA nutritional supplementation and prevention of cardiovascular diseases. The results demonstrate that the membrane FA composition affects several parameters of monocyte apoptosis, including DNA fragmentation, caspase-3 activity and expression, mitochondrial potential $\Delta\Psi_m$ and cardiolipin content, both under basal conditions (native LDL) and under stimulated conditions (HOCl-oxLDL).

Hazell *et al.* demonstrated that HOCl, a natural, non-metal-dependent and powerful oxidant, causes modification of proteins in human atherosclerotic lesions *in vivo*[20, 21]. Since they suggested that this oxidant may contribute significantly to atherosclerosis, we decided to use HOCl-oxLDL in our study. Moreover, this form of oxLDL model is considered to be the most relevant form of lipoprotein alteration under atherosclerotic pathophysiological conditions[22-24]. Vicca *et al.* studied the apoptotic signaling pathway occurring in the U937 model[9], and a recent complementary study reported the involvement of the mitochondrial caspase-dependent pathway after HOCl-ox-LDL treatment of U937, resulting from reactive oxygen species (ROS) generation and Bax translocation[25]. PUFA are considered as a potential target of oxidative stress because of the high number of double bonds sensitive to peroxidation, and the resulting lipoperoxides were suggested to be potential mediators of apoptosis[26]. In a recent work, Tardivel *et al.* reported the generation of ROS in DHA- enriched endothelial cells[27], and interestingly, these results as well as those of the present study outline the possibility of a key role of ROS in monocyte apoptosis after membrane PUFA enrichment. Here, we report a significant pro-apoptotic effect with either EPA (C20:5 n-3) or ARA (C20:4 n-6) in U937 monocytes, whereas supplementation with either DHA or OA did not affect apoptosis. The different parameters investigated in this study (DNA fragmentation, caspase-3 activity, $\Delta\Psi_m$ and mitochondrial cardiolipin) were significantly altered by

EPA or ARA, both under basal conditions and HOCl-oxLDL- stimulated conditions. Conversely, DHA or OA supplementation did not alter these parameters (as compared to control cells) under conditions basal or after HOCl-oxLDL. Since cardiolipin depletion could precede the release of mitochondrial cytochrome c, our data suggest that EPA or ARA induced apoptosis through a mitochondrial effect on U937 cells. Moreover, the results obtained with U937/Bcl-2 cells strongly support the importance of the mitochondrial pathway of apoptosis. Indeed, the Bcl-2 overexpression led to the inhibition of several mitochondrial apoptotic activities and rescued cells from apoptosis by maintaining membrane integrity[25].

Interestingly, the analysis of membrane lipid composition demonstrated a high 20-carbon PUFA content after EPA or ARA treatment (28.2% and 29.7% for EPA-treated cells and ARA- treated cells), whereas these PUFA remained low in the 3 other groups (9.1%, 7.3% and 5.7% in control, DHA- and OA- treated cells, respectively). The fact that only these membrane PUFA act as substrates for cyclooxygenase and lipoxygenase suggests an increase in the total level of oxygenation processes. As evidenced by the similar effect of EPA and ARA (but not DHA) the present results do not point out the importance of n-3 vs n-6 PUFA, which have controversial effects since a high n-6/n-3 ratio was reported to promote CVD[28, 29]. On the contrary, they point out a specific pro-apoptotic effect of 20-carbon PUFA regardless of the series. In a recent review, Arnold *et al.*[30] reported that some of the cardiovascular effects attributed to dietary n-3 and n-6 PUFAs may be mediated by cytochrome P450-dependent metabolites. Indeed, EPA, ARA, and DHA are converted to epoxy- and/or hydroxy-fatty acids by CYP450-linked mono-oxygenase and may have far-reaching physiological implications. The results of Oliw *et al.*[31] indicated that the n-3 hydroxylase and n-3 epoxygenase enzymes metabolize ARA and EPA almost exclusively to the n-3(R) alcohol and the n-3(R, S) epoxide, respectively, while longer or shorter fatty acids (such as DHA or OA) are either poor substrates or are metabolized with a lesser degree of position specificity. Thus, ARA and EPA, two 20-carbon PUFA, could exert pro-apoptotic effects in relation with their epoxy or hydroxy metabolites.

It is generally admitted that apoptosis is harmful in advanced atherosclerotic lesions[10, 32, 33] but its role in the early stage of atherosclerosis, particularly during monocyte infiltration, is not well defined. Matsumoto *et al.*[34] recently reported that orally administrated EPA may reduce and stabilize atherosclerotic lesions in apolipoprotein E knock-out (ApoE-/-) mice through its anti-inflammatory properties. Similarly, Xu *et al.* did not observe any modulation of atherogenesis by EPA or DHA in ApoE-/- mice fed 1% fish oil[35]. The absence of an EPA effect at low intake may be due to the specific incorporation of this FA into monocyte phospholipids, largely lower *in vivo* than *in vitro* in U937 cell culture. Negative results obtained *in vivo* by Xu *et al.* were explained by the lack of ApoE in the mouse model and the resulting lack of ApoE-mediated uptake of triglyceride-rich particles, which could be involved in the physiological effects of FA.

In conclusion, EPA or ARA cell membrane overloading exerts a significant pro-apoptotic effect *in vitro* in U937 monocytes added to the synergic apoptotic effect of oxLDL, whereas DHA and OA have no effect. Interestingly, in agreement with our results, EPA and ARA were also described as potent apoptosis inducers in human monocytes[36, 37]. This raises the hypothesis of a specific effect of PUFA with 20 carbons, independently of the series involved (n-6 or n-3) and supports the role of inflammatory processes since these PUFA are both involved in prostaglandin and leukotriene pathways. Moreover, further nutritional studies are needed to underline the clinical relevance of this work in CVD prevention. Recently, Mebarek *et al.* investigated the effects of increasing doses of DHA added to the regular diet of human healthy volunteers on the apoptosis of monocytes isolated from peripheral blood[38]. Interestingly, the authors showed that low DHA doses increased monocyte resistance to apoptosis, which might be beneficial in the prevention of CVD. The characterization of mechanisms potentially involved in cell death regulation is still to be defined to better understand the physiopathology of atherosclerosis and to develop new nutritional strategies that could prevent atherosclerosis progression.

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