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Mechanisms of Cell Death of Thymocytes Induced by Polyunsaturated, Monounsaturated and Trans-Fatty Acids

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) are rapidly cytotoxic to isolated murine thymocytes, and the degree of cell death has been correlated with changes in membrane fluidity, elevation of intracellular calcium concentration and generation of reactive oxygen species. We have compared the degree of cell death and increase in membrane fluidity of C-20 and C-22 omega-3 and 6 PUFAs to those induced by monounsaturated and trans-fatty acids, and find that concentrations which induce comparable increases in membrane fluidity do not cause comparable cell death. The C-18 omega-6 causes a decrease in membrane fluidity, yet is the most potent in causing cell death. Omega-6 PUFAs are more cytotoxic than omega-3 PUFAs, while monounsaturated and trans-fats show little cytotoxicity and only at much higher concentrations. Cell death is preceded by reductions of both plasma and mitochondrial membrane potential, and occurs via apoptosis. These results indicate that cell death is due to mechanisms other than changes in membrane fluidity. J. Cell. Biochem. 112: 3863–3871, 2011.

KEY WORDS: OMEGA-3 FATTY ACIDS; APOPTOSIS; MITOCHONDRIA; MEMBRANE FLUIDITY; CALCIUM

Omega-3 polyunsaturated fatty acids (PUFAs) are essential to humans and have a variety of biochemical, nutritional and epigenetic properties [Benatti et al., 2004]. They are major components on neuronal membranes [Horrocks and Farooqui, 2004], and deficiency results in impaired learning and visual function [Bourre et al., 1993]. They are also thought to be important for cardiovascular health [Hooper et al., 2006] and are known to reduce risk of sudden cardiac death following a heart attack [Albert et al., 1998]. The mechanism is believed to be that they become incorporated into cardiac muscle membranes, increasing membrane fluidity and as a result reducing entry of sodium and calcium into cells [Leaf et al., 2005]. In contrast, saturated and trans-fats increase risk of cardiovascular disease [Oh et al., 2005; Woodside and Kromhout, 2005]. Omega-6 PUFAs are also essential, but these PUFAs are rarely lacking in the current human diet, leading some to propose that the omega-6 to omega-3 PUFA ratio has important implications for human health [Harbige, 2003; Wijendran and Hayes, 2004; Simopoulos, 2008].

It has been known for many years that exogenous lipids can alter membrane fluidity. Saturated fats [Calder et al., 1994] and cholesterol [Lau and Das, 1995] cause a decrease in fluidity, while omega-3 fatty acids cause an increased fluidity in various cells [Hashimoto et al., 1999, 2001] and membranes including mitochondrial membranes [Stillwell et al., 1997]. Arachidonic acid (C-20, omega-6) (ARA) has also been reported to increase fluidity of endothelial cells [Villacara et al., 1989] and to help

Abbreviations: ARA; 20:5n-6, arachidonic acid; ALA; 18:3n-3, α linolenic acid; BAPTA; 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; C; carbon; CCCP; carbonyl cyanide 3-chlorophenylhydrazone; DCF; dichlorofluorescein; $DiBAC_4(3)$; bis-(1; 3-dibutylbarbituric acid)trimethine oxonol; DPH; 1,6-diphenyl-1,3,5-hexatriene; DHA; 22:6n-3; docosahexaenoic acid; DilC1(5); 1,1́,3,3,3́-hexamethylindodicarbocyanine idodide; DTA; 22:4n-6; docosatetraenoic acid; EA; t18:1; elaidic acid; EPA; 20:5n-3; eicosapentaenoic acid; HEPES; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LNA; 18:2n-6; linoleic acid; MMP; mitochondrial membrane potential; MUFA; monounsaturated fatty acids; OLA; oleic acid; P; fluorescence polarization; PI; propidium iodide; PMP; plasma membrane potential; PUFAs; polyunsaturated fatty acids; ROS; reactive oxygen species; SEM; standard error of the mean.

Mari Åhs and Aparna Prasad contributed equally to this study.

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maintain neuronal function in old rat neurons by reversing the age-dependent reduction in membrane fluidity [Fukaya et al., 2007]. Changes in membrane fluidity have been shown to alter binding, insertion, and activity of enzymes [Pande et al., 2005].

However, omega-3 PUFAs also cause acute cytotoxicity in several types of cells [Finstad et al., 1998; Andrade et al., 2005]. This is a consequence of rapid incorporation of the PUFAs into the membrane with a subsequent alternation of structure and function [Zerouga et al., 1996]. Whether the cytotoxicity is due to the change in fluidity remains uncertain.

In studies of acutely isolated murine thymocytes, we have shown that both omega-3 and omega-6 PUFAs are acutely cytotoxic and that the cytotoxicity is associated with changes in membrane fluidity [Prasad et al., 2010a]. We found that for comparable chain length omega-6 PUFAs were more potent than omega-3 PUFAs and that the relative cytotoxicity of the C-20 and C-22 omega-3 and omega-6 PUFAs was correlated with the degree of increase in membrane fluidity. However, the most cytotoxic PUFA (linoleic acid, C-18 omega-6) (LNA) caused a decrease, not an increase, in membrane fluidity. In a subsequent study [Prasad et al., 2010b], we found that cell death after exposure to either omega-3 or 6 PUFAs was dependent upon first a release of calcium from endoplasmic reticulum, followed by generation of reactive oxygen species (ROS) from mitochondria, and that this was true whether or not membrane fluidity was increased or decreased. We concluded that cell death was immediately dependent on the production of ROS, but assumed, based on our earlier report [Prasad et al., 2010a], that the ROS generation was dependent upon the change in membrane fluidity.

The present studies are extensions of our past work, and are directed at a comparison of monounsaturated and trans-fats with PUFAs in terms of effects on membrane fluidity and cytotoxicity, as well as more in depth analysis of the mechanisms responsible for cell death. We provide evidence that the change in fluidity is independent of cell death, which is due to apoptosis.

MATERIALS AND METHODS

PREPARATION OF THYMOCYTES

The thymus gland was acutely removed from 4- to 6-week-old ICR mice (Taconic Farms Inc., Germantown, NY) in experiments reviewed and approved by the Institutional Animal Care and Use Committee of the University at Albany. The thymus was then cut into slices, and thymocytes dissociated by grinding between two frosted microscope slides in cold Tyrode's buffer (148 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose at 7.4 pH) as previously described [Oyama et al., 1995]. The extracted cells were then filtered through a cell strainer (70 μm) and washed with Tyrode's buffer. The single cell suspension was adjusted to a concentration of approximately 1×10^6 cells/ml in Tyrode's buffer for experiments.

CHEMICALS AND REAGENTS

Propidium iodide (PI) was purchased from Molecular Probes Inc. (Eugene, OR). Sodium phosphate monobasic ($NaH_2PO_4.H_2O$) and sodium phosphate dibasic anhydrous (NaH_2PO_4) were bought from Fisher Scientific (Fairlawn, NJ). Bis-(1, 3-dibutylbarbituric acid)-trimethine oxonol [$DiBAC_4(3)$], mitoprobe DilC1 (5), and 5,6-carboxy-2'7'-dichlorofluorescein diacetate (Carboxy-DCFDA) were purchased from Invitrogen (Eugene, OR). Annexin V-FITC was purchased from Sigma–Aldrich (St. Louis, MO). Sodium phosphate monobasic ($NaH_2PO_4.H_2O$) and sodium phosphate dibasic anhydrous (NaH_2PO_4) were purchased from Fisher Scientific (Fairlawn, NJ). The other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). In all the experiments we used bi-distilled water. Fatty acids were dissolved in ethyl alcohol so that the final concentration was never >0.2%. No effect of 0.2% ethyl alcohol on any of the measured parameters was detected.

The fatty acids studied include three omega-3 PUFAs docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and α linolenic acid (ALA, 18:3n-3). Omega-6 PUFAs were docosatetraenoic acid (DTA, 22:4n-6), arachidonic acid (ARA, 20:5n-6) and linoleic acid (LNA, 18:2n-6). In addition, we studied oleic acid (OLA, 18: 1n-9), a monounsaturated fatty acid, and elaidic acid (EA, t18:1), a common trans-fat.

FLUORESCENCE POLARIZATION

Membrane fluidity was determined by measurement of fluorescence polarization with a Perkin Elmer LS50 fluorescence spectrophotometer using 1,6-diphenyl-1,3,5-hexatriene (DPH) to detect the dynamic movements (rotational and transactional movement) of the lipids in the thymocyte membrane [Chen et al., 1998]. DPH has the shape of a cylinder and a fluorescence emission transition dipole aligned approximately parallel to its long molecular axis [Lasner et al., 1995]. Because of its lipophilic nature DPH inserts into the membrane with its long axis parallel to the fatty acid chains. If membrane fluidity increases, the molecules change the orientation of its long axis and this is detected as a decrease in fluorescence polarization, while if fluidity decreases the fluorescence polarization will increase. A stock solution of DPH was made in tetrahydrofuran at 1 mM. Cells and the buffer were mixed with 4 μM of DPH and this solution was then incubated at room temperature in darkness for 45 min. The excitation and emission wavelength were used at 360 and 430 nm, respectively.

The effect of fatty acids on steady-state fluorescence polarization (P) was calculated by using $P = (I_{vv} - gI_{vh})/(I_{vv} + gI_{vh})$, where g is a grating correction factor, equal to Ivh/Ihh. I is the light intensity, and v and h stand for vertically and horizontally oriented polarizers [Lasner et al., 1995; Chen et al., 1998]. The first and second subscript refers to the excitation and emission light, respectively.

Each data (P) represented an average of 10 measurements.

FLOW CYTOMETRY

A BD LSRII Flow Cytometer (BD Biosciences, San Jose, CA) was used to measure cell death, plasma membrane potential (PMP), mitochondrial membrane potential (MMP), and apoptosis. The DNA-binding dye, PI was used to measure cell death. After incubating the cells with fatty acids, 10 μM PI was added 3–5 min before measurement. In healthy cells, PI is excluded by the plasma membrane, but in cells with compromised membrane integrity it enters into the cell and binds to DNA. PI fluorescence of live cells is low but in cells with compromised membrane integrity it increases

by about four orders of magnitude. Each flow cytometry experiment was repeated at least five times on separate days.

The fluorescent dyes $DiBAC_4$ (3) and DilC1 (5) were used to measure plasma and MMP, respectively. $DiBAC_4$ (3) enters depolarized cells where it binds to intracellular proteins or membrane and shows enhanced fluorescence. Cells were incubated with $DiBAC_4$ (3) (50 nM) for 20 min in the dark. Cells were further exposed to different fatty acids for 1 h and $DiBAC_4$ (3) fluorescence was measured. For some experiments $DiBAC_4$ (3)-loaded cells were pretreated with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) (5 μM) for 30 min at 37°C in the dark, washed and resuspended in buffer.

DilC1 (5) accumulates primarily in mitochondria with active membrane potential. DilC1 (5) stain intensity decreases when cells are treated with chemical like carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (2 μM), a proton ionophore which uncouples electron transport and disrupts MMP. Thymocytes were loaded with DilC1 (5) (50 nM) for 15–30 min at 37°C in the dark. The cells were then washed and resuspended in Tyrode's buffer. Cells were further exposed to different fatty acids for 1 h and DilC1 (5) fluorescence was measured.

The cellular content of ROS was determined using carboxy-DCF-DA. Carboxy-DCF-DA readily enters into cells and its acetate groups are cleaved by intracellular esterase enzymes, thus converting DCF-DA to DCF-H2. After cleavage DCF-H2 is retained inside the cell. In the presence of ROS, DCF-H2 is oxidized and converted into a fluorescent molecule. The isolated thymocytes were loaded with carboxy-DCF-DA (100 μM) for 45 min in the dark. The cells were then washed two times and resuspended in Tyrode's buffer. DCF-loaded cells were pretreated with CCCP (2μM) for 30 min at 37°C in the dark. Cells were further exposed to different fatty acids for different times and DCF fluorescence measured.

Annexin V-FITC was used to detect apoptotic cells through flow cytometry. Annexin binds to phosphatidylserine residues, which under normal circumstances are on the inside leaflet of the plasma membrane, but which move to the outer leaflet early in of the process of apoptosis. Cells were treated with different fatty acids for 1 h at 37°C in the dark and washed with Tyrode's buffer and resuspended in buffer at a concentration of $\sim 1 \times 10^6$ cells/ml. Cells were treated with Annexin V FITC conjugate (5 μl) and PI and incubated at room temperature for 10 min in dark. Fluorescence of the cells was then determined. Cells which are early in the apoptotic process will stain only with the Annexin V FITC Conjugate. Live cells will show no staining. Dead cells will be stained by both the PI and Annexin V FITC conjugate, but cells dying via necrosis will not show the intermediate staining with Annexin V.

After mixing cells with different fluorescent dyes, cells were excited with an argon laser (488 nm) and 10,000 cells were analyzed per sample. The emission wavelengths for PI was 575 nm and for $DiBAC_4$ (3) and Annexin-V was 525 nm. The excitation and emission wavelengths for DilC1 (5) was 633 and 658 nm, respectively. Since the emission spectra for PI and $DiBAC_4$ (3) or DilC1 (5) or Annexin-V did not overlap it was possible to measure fluorescence of PI and each of the other dyes simultaneously. Depending on forward scatter (an indicator of cell size) and side scatter (an indicator of cellular granularity), a relatively homogeneous subpopulation of total cells (>90%) was gated for analysis. Data were analyzed by using BD FACSDiva Software.

STATISTICS

All results are presented as means with SD. Independent sample *t*-tests were used to compare different concentrations of PUFAs and different incubations times. One-way ANOVA with post hoc Tukey test was used to analyze fluorescence polarization and cell viability when comparing different PUFAs. Results were considered significant if $P < 0.05$.

RESULTS

EFFECT OF OMEGA-3, OMEGA-6, MONOUNSATURATED AND TRANS-FATTY ACIDS ON CELL DEATH AND MEMBRANE FLUIDITY

Figure 1a shows the degree of cell death of thymocytes caused by incubation at two concentrations and two periods of time by an omega-3 (EPA), omega-6 (ARA), trans- (EA), and monounsaturated (OLA) fatty acids. These results show that the C-20 omega-6 fatty acid is significantly more cytotoxic than the omega-3 at comparable concentrations and periods of time, whereas much higher concentrations of trans- and monounsaturated fatty acids cause less toxicity even when incubated for a longer period of time. Figure 1b shows the reduction in fluorescence polarization, indicative of increases in membrane fluidity, induced by these same applications. All four fatty acids trigger an increase in membrane fluidity of nearly the same magnitude, increasing slightly with time. There were no effects of EA or OLA at sub-millimolar concentrations on either cell death or membrane fluidity. These observations indicate that the degree of cell death is not well correlated with the changes in membrane fluidity either upon consideration of which fatty acids was applied or for what period of time.

EFFECTS OF OMEGA-3 AND 6 FATTY ACIDS ON CELL DEATH, AND ON PLASMA AND MITOCHONDRIAL MEMBRANE POTENTIAL

Figure 2 shows results of application of three omega-3 fatty acids, DHA, EPA, and ALA (each at 20 μM) on cell death (a) and PMP (b). DHA was the most potent fatty acid, followed by EPA, in inducing cell death. Pretreatment of cells with BAPTA-AM, an intracellular calcium chelator, reduced omega-3-induced cell death, indicating a role of intracellular calcium. $DiBAC_4$ (3) is an anionic oxonol dye which responds with an increase in fluorescence intensity upon plasma membrane depolarization. The degree of membrane depolarization was proportional to the relative cytotoxicity, and was prevented by application of BAPTA. Thus, omega-3-induced cell death and membrane depolarization are dependent on an elevation of intracellular calcium concentration.

Similar experiments were done to see the effect of omega-6 fatty acids on cell death and PMP. LNA was the most potent, but DTA was more potent than ARA in cytotoxicity (Fig. 2c) and PMP depolarization (Fig. 2d). As with omega-3s, omega-6 induced increase in cell death and PMP depolarization was dependent on calcium.

DilC1 (5) is a cationic cyanine dye which accumulates in mitochondria as a function of MMP. In contrast to $DiBAC_4$, DilC1 (5) fluorescence intensity decreases when MMP is reduced. CCCP is a

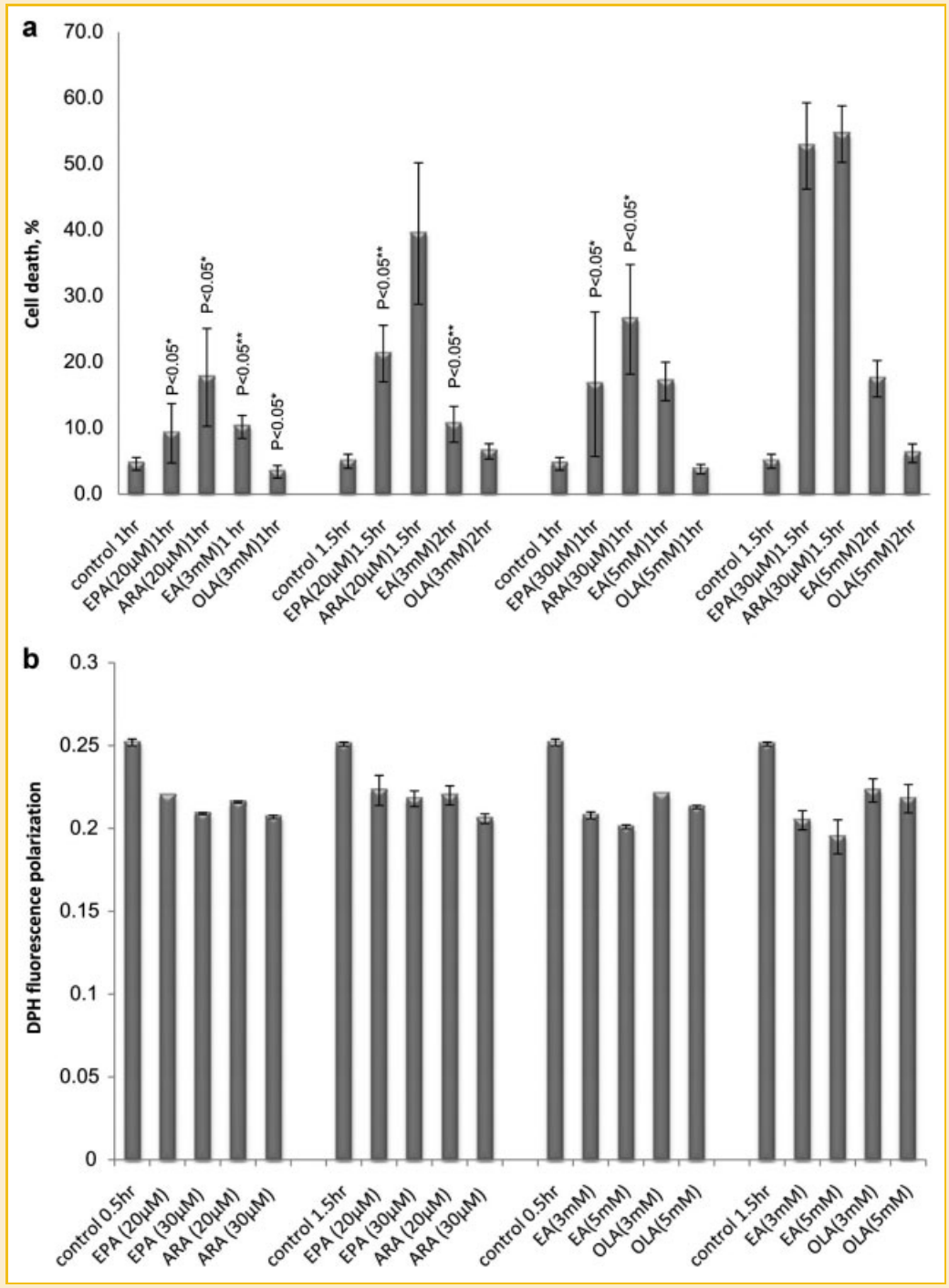

Fig. 1. Effects of EPA (20 and 30 μM), ARA (20 and 30 μM), EA (3 and 5 mM) and OLA (3 and 5 mM) on cell death at 1 and 1.5 hr (a) and membrane fluidity after 0.5 and 1.5 hr (b), measured by DPH fluorescence. Each bar is the average of results from 10 experiments. Those values that are significantly different from control are indicated by $P < 0.05^{*}$; significantly different values on comparison of various doses are indicated by $P < 0.05^{**}$. (a) There are significant differences in the values for EPA, ARA and OLA as a function of time at both concentrations except for 5 mM OLA. There were no significant differences for EA as a function of time, but values were significantly higher for 5 mM in both cut points of time (1 and 1.5 hr). (b) All data are significantly different from control, but not from each other.

proton ionophore that uncouples electron transport in mitochondria and causes collapse of the MMP. Figure 3 shows the effects of CCCP (2 μM), omega-3 fatty acids (a) and omega-6 fatty acids (b) on DilC1 (5) fluorescence. CCCP caused a reduction in DilC1 (5) fluorescence, as expected. The omega-3 and 6 fatty acids also caused a reduction in fluorescence in proportion to their relative cytotoxicity. These findings suggest that mitochondrial membrane depolarization is a general component in PUFA-mediated cell death. The effects of CCCP on EA and OLA responses were not studied because the degree of cell death was so small.

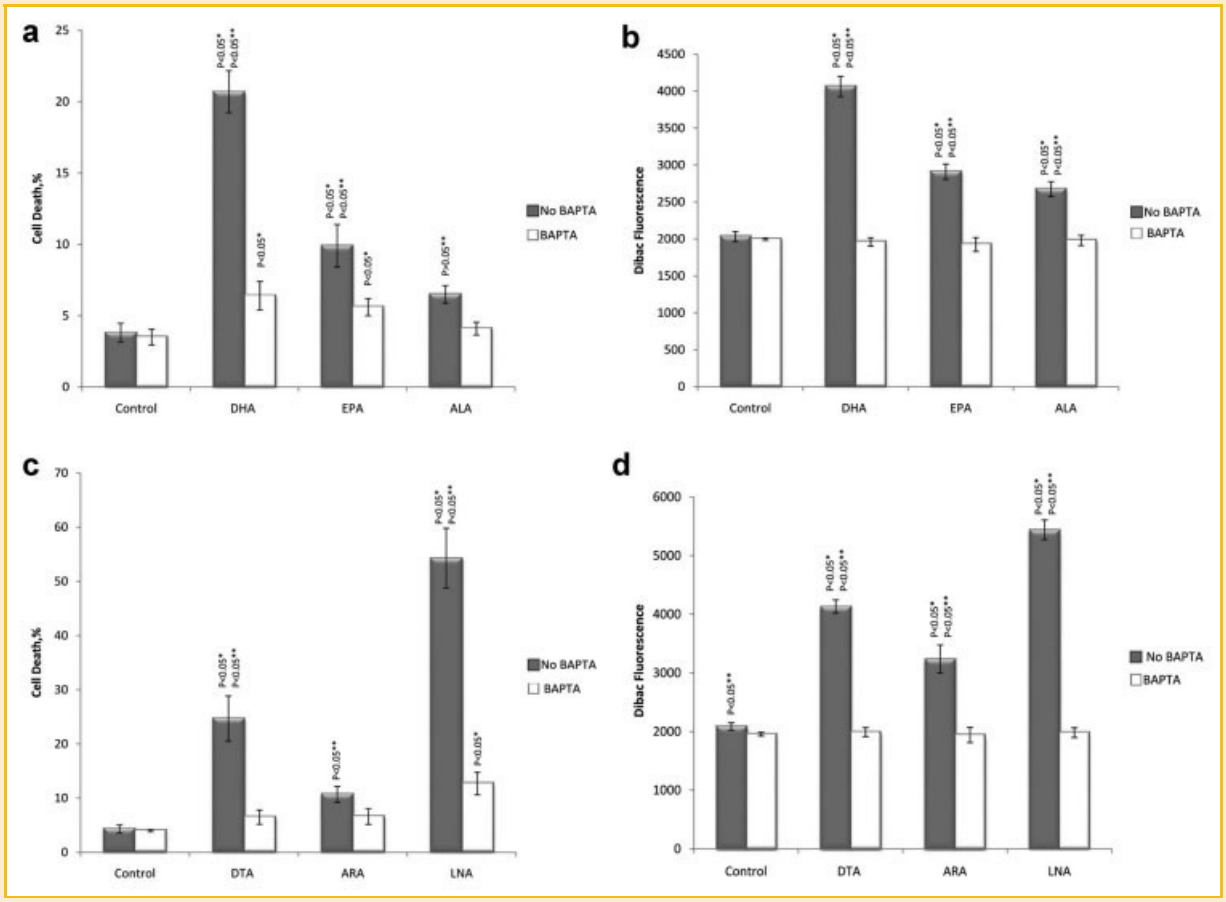

Fig. 2. Effects of the omega-3 fatty acids, DHA, EPA and ALA, and omega-6 fatty acids, DTA, ARA and LNA, on cell death (a,c) and PMP (b,d) as measured by PI and $DiBAC_4$ fluorescence, respectively, in the presence and absence of BAPTA (5 μM). Cells were incubated with $DiBAC_4$ (50 nM) for 20 min, then with the fatty acids (20 μM for omega-3s; 10 μM for omega-6s) for 1 h. PI (10 μM) was added 3–5 min before measurements were taken. When BAPTA was used it was added 30 min before the fatty acids. Those values not significantly different from control are indicated by $P > 0.05$. For cell death not with BAPTA (a), DHA is significantly different from EPA which is significantly different from ALA, but in the presence of BAPTA only DHA is significantly different from ALA. b: DHA is significantly different from EPA and ALA, but EPA and ALA are not significantly different from each other in absence of BAPTA. c: There are significant differences between DTA, ARA, and LNA in the absence of BAPTA, but in the presence of BAPTA only LNA is significantly different from DTA and ARA. d: DTA, ARA, and LNA are all significantly different from each other in absence of BAPTA.

EFFECT OF OMEGA-3 FATTY ACIDS AND CCCP ON ROS PRODUCTION

We have previously shown that PUFA-induced cytotoxicity was dependent upon production of ROS, and we hypothesized that the source was mitochondria. To confirm this we investigated ROS production using DCF in the absence and presence of CCCP. Concentrations of omega-3 fatty acids that cause cell death were associated with proportional elevations in DCF fluorescence, and this fluorescence was greatly reduced in the presence of CCCP (Fig. 4). Similar results were obtained with omega-6 PUFAs (data not shown).

FATTY ACID-INDUCED CELL DEATH IS DUE TO APOPTOSIS

Figures 5 and 6 provide support for the conclusion that cell death resulting from acute application of fatty acids onto thymocytes is due to apoptosis. Figure 6 shows dot plots of changes in fluorescence to PI as compared to Annexin-V. Healthy cells are in quadrant 3, being low in both PI and Annexin-V. Cells undergoing apoptosis will show Annexin-V staining at a time when membrane integrity is intact, and will therefore move to quadrant 4. Cells undergoing necrosis will move quickly to quadrant 2 but through quadrant 1. When membrane integrity is lost, both PI and Annexin-V will enter and the cells will be labeled with both (quadrant 2). It is apparent in Figure 5 that the three omega-3 fatty acids all cause cell death via apoptosis, and that the relative degree of cell death is consistent with that shown in Figure 2.

Figure 6 shows the results of a similar study of EPA, ARA, EA, and OLA after a 1.5-h incubation, and gives a plot of the percentage of cells located in quadrant 4 (Annexin V positive but PI negative) and quadrant 2 (positive for both indicating loss of membrane integrity). These results are consistent with the conclusion that cell death from exposure to EPA and ARA is via apoptosis, but that EA and OLA are much less toxic.

DISCUSSION

PUFAs, both omega-3 and omega-6, are known to modulate immune system function in multiple ways [Calder, 2007]. While in

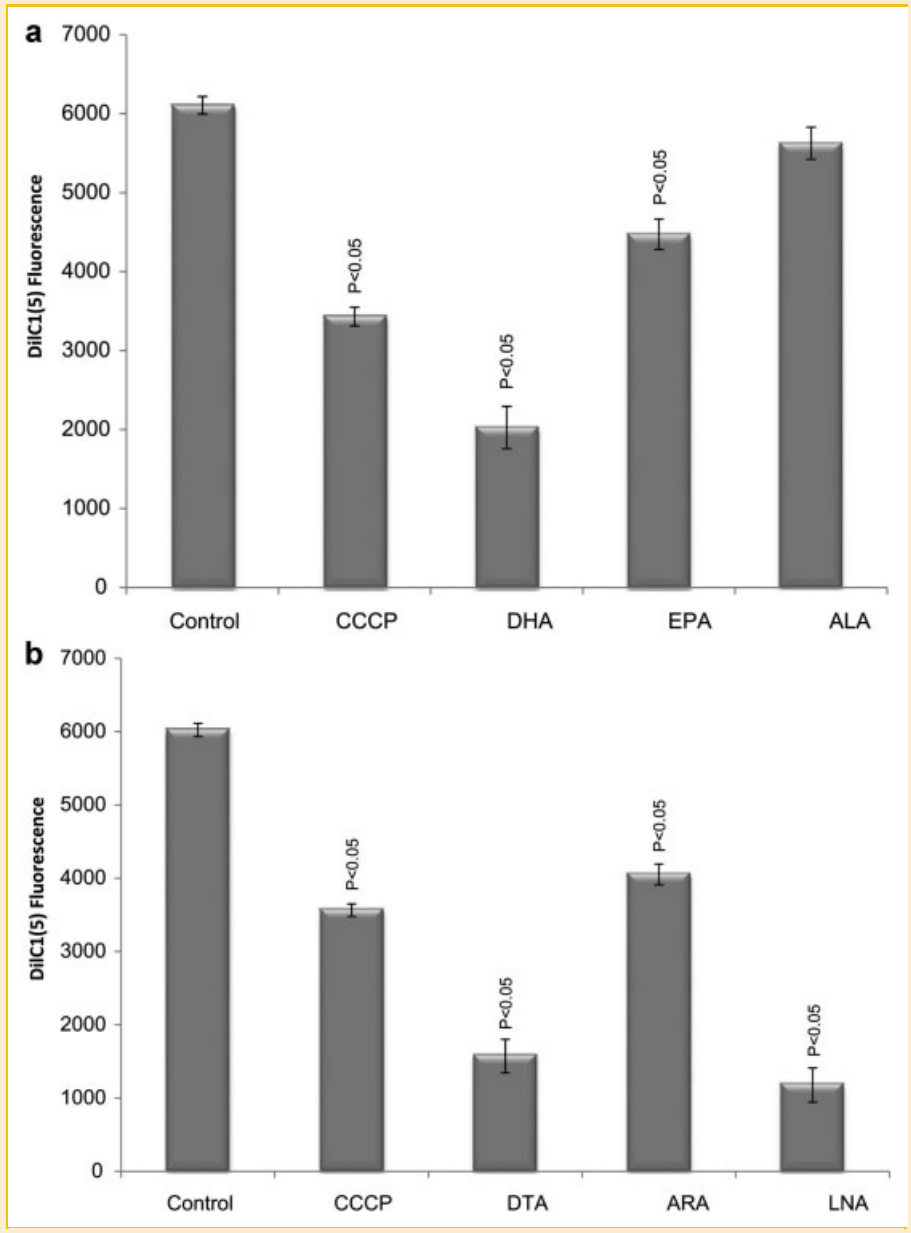

Fig. 3. Effects of CCCP (2 μM) and CCCP plus omega-3 (20 μM) (a) and 6 (10 μM) (b) fatty acids on mitochondrial membrane potential (MMP) as measured by DilC1(5). Cells were incubated with DilC1(5) (50 nM) for 15–30 min, then the PUFAs added for 1 h. When CCCP (2μM) was used it was added 30 min before the fatty acids. a: ALA is not significantly different from control, but DHA, EPA, and ALA are significantly different from each other and from CCCP. b: All are significantly different from control and the PUFAs are all significantly different from CCCP. DTA and LNA are not significantly different from each other.

general omega-3s are anti-inflammatory and omega-6s are pro-inflammatory, both types of PUFAs have multiple actions and give rise in other active agents in the intact organism. Omega-3 PUFAs have been reported to suppress T-cell activation by displacing proteins from lipid rafts [Fan et al., 2003], and both omega-3 and omega-6 PUFAs are clinically useful in treating autoimmune and inflammatory diseases [Harbige, 2003].

It is somewhat counterintuitive that essential fatty acids that are important to immune system function would be cytotoxic to thymocytes at quite low concentrations. While these are not physiologic conditions, as sudden increases in the concentrations of omega-3 or 6 fatty acids of this magnitude in serum are unlikely to occur in an intact person or animal, these studies do provide information on important physiologic events the occur as a consequence of changes in membrane structure.

Increases in membrane fluidity from long-chain PUFAs have been demonstrated in a variety of types of cell, including lymphocytes [Calder et al., 1994], human erythrocytes [Lund et al., 1999], and endothelial and vascular smooth muscle cells [Beck et al., 1998]. It has been proposed that the beneficial actions of omega-3 fatty acids in preventing sudden cardiac death in human is secondary to the alterations in sodium [Xiao et al., 1995] and

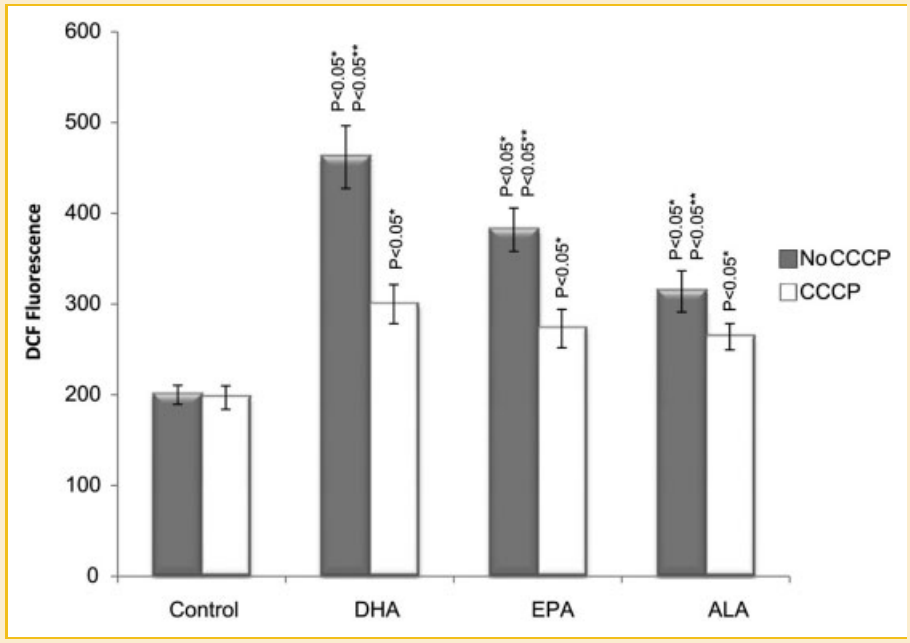

Fig. 4. Effects of omega-3 fatty acids (20 μM for 1 h) on ROS production in the presence and absence of CCCP. Cells were incubated with 100 μM carboxy-DCFDA for 45 min before adding the fatty acids (20 μM) for 1 h. When CCCP (2 μM) was used it was added 30 min before the fatty acids. All results are significantly different from control and in absence of CCCP, and DHA, EPA, and ALA are all significantly different from each other. In the presence of CCCP, all values are significantly different from control but not from each other.

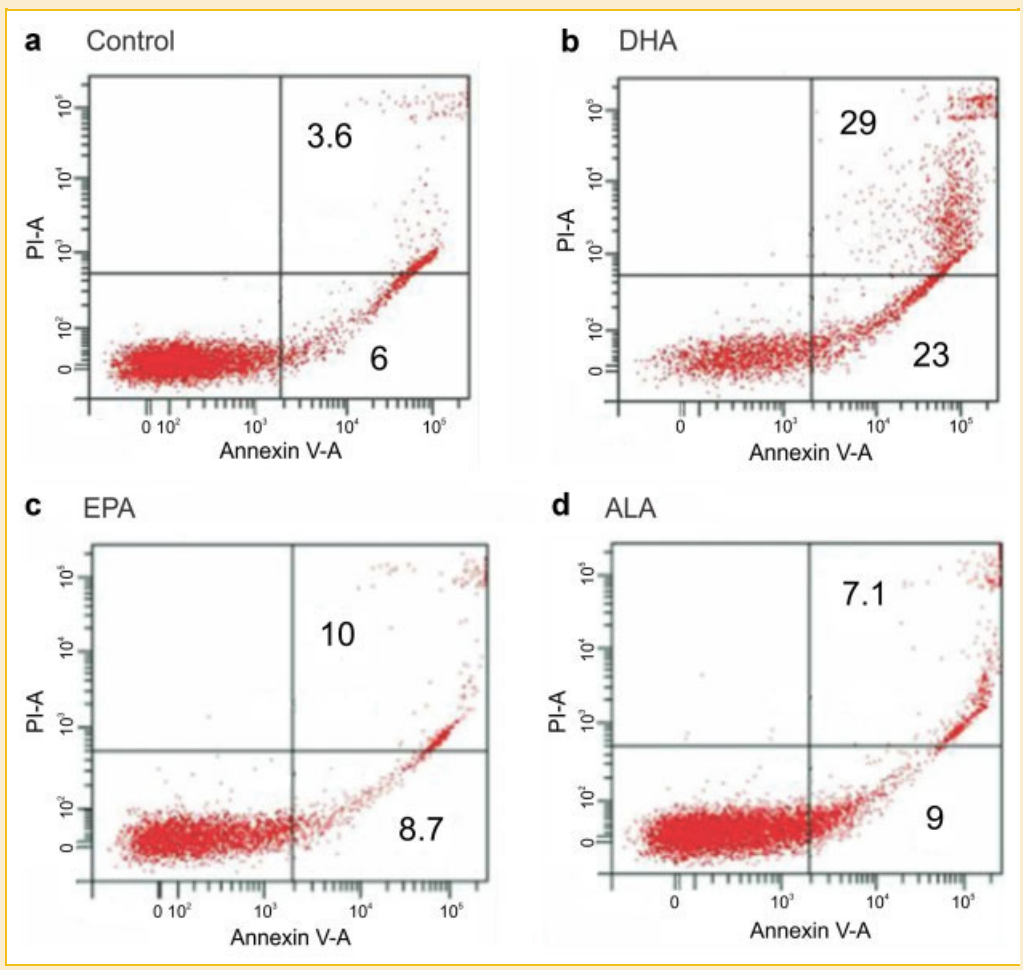

Fig. 5. Omega-3 fatty acids cause cell death via apoptosis. Cells were incubated with DHA, EPA or ALA (20 μM) for one hour, washed and then exposed to Annexin V FITC conjugate (5 μl) and PI (10 μM) for 10 min before measurement.

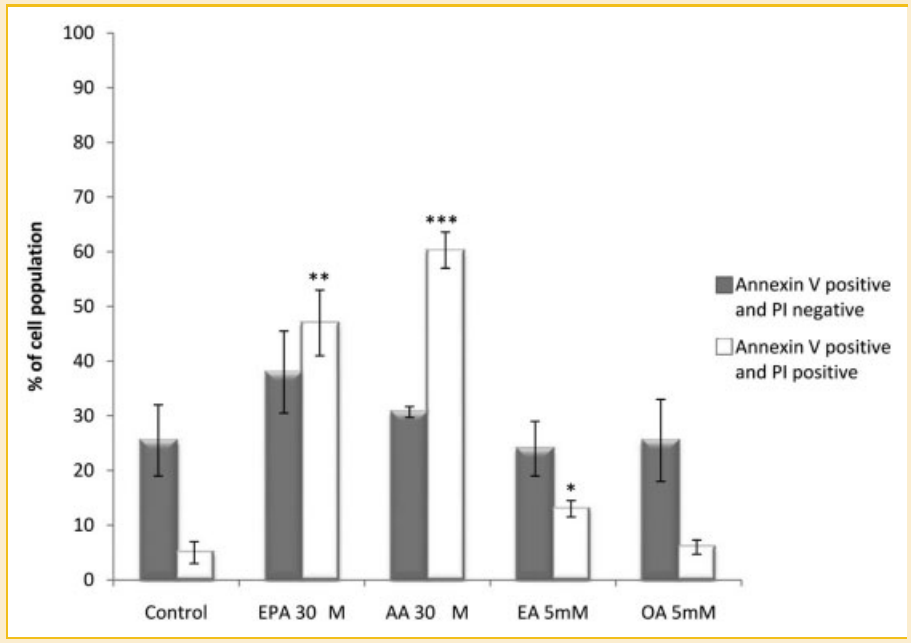

Fig. 6. Effects of EPA, ARA, EA, and OLA at the concentrations given for 1.5 h on early apoptosis (Annexin V positive and PI negative) and cell death (Annexin V positive and PI positive). $^{*}P = 0.008$; $^{**}P = 0.00015$, $^{****}P = 0.00003$ relative to control.

calcium [Xiao et al., 1997]. These effects were demonstrated at PUFA concentrations in the low micromolar range, and were attributed to increased fluidity in the micro domains of the cell membrane [Leaf and Xiao, 2001]. Although our studies are on a different cell type, the concentrations of PUFAs that alter membrane fluidity are similar to those reported to alter ion channel activities.

Our previous report [Prasad et al., 2010a] attributed the cell death from omega-3 and 6 fatty acids to be a result of the changes in membrane fluidity on the basis of the observation that the viability and fluidity changes were correlated in time and with concentration. However, in these experiments we find comparable changes in fluidity from exposure to higher concentrations of monounsaturated and trans-fatty acids. But the increase in fluidity caused by the monounsaturated and trans-fats was not associated with significant effects on cell death and occurred only at concentrations two orders of magnitude higher that that for the PUFAs. These results suggest that the fluidity changes are not the immediate cause of the cell death.

A more detailed study into mechanisms found that cell death could be prevented by both preventing a rise in intracellular calcium and/or ROS concentrations [Prasad et al., 2010b]. We concluded that the initial event was release of calcium from endoplasmic reticulum, and that the elevated intracellular calcium caused generation of ROS, presumably from mitochondria. Because ROS scavengers blocked cell death but not the elevation of intracellular calcium concentration, we concluded that it was the increased levels of ROS that caused the cell death. The demonstration in the present report that both PMP and MMP are reduced by PUFAs in relation to their level of cytotoxicity, and that these effects on membrane potential are blocked by intracellular BAPTA, is consistent with the evidence that release of calcium from endoplasmic reticulum is a critical first step. Finally, the demonstration that CCCP (which depolarizes mitochondria) reduces DCF fluorescence is consistent with mitochondria being the major source of the ROS. CCCP did not totally block the elevation in DCF fluorescence induced by the omega-3 PUFAs, which indicates either that inhibition of electron transport is not complete or that there are minor non-mitochondrial sources of ROS as well. The depolarization induced by CCCP did not cause rapid cell death, which indicates that the mechanism of action of PUFAs is due to more than just mitochondrial depolarization. We assume that when PUFAs are incorporated into the mitochondrial membrane they alter some critical function that is not changed by either just a change in fluidity or just depolarization of MMP. We did not do detailed studies of effects of CCCP on EA or OLA because they were only weakly cytotoxic and only at very high concentrations.

Our results provide evidence that the mechanism of cell death is via apoptosis. There have been a number of reports that omega-3 fatty acids induce apoptosis in a variety of lymphoma [Finstad et al., 1998a] and leukemia [Finstad et al., 1998b] cell lines. Some [Nasrollahzadeh et al., 2009] have even suggested that DHA might be useful as an anti-carcinogenic therapeutic drug. However, our results show clearly that the induction of apoptosis is not limited to cancer cells, which makes it unlikely that they would be useful for this purpose.

Omega-3 fatty acids are widely believed to have many beneficial effects, particularly in preventing cardiovascular [Kris-Etherton and Harris, 2002] and neurologic [Horrocks and Farooqui, 2004] diseases They also have net anti-inflammatory actions on the immune system, while the omega-6 fatty acids are predominately pro-inflammatory [Harbige, 2003]. The detailed mechanisms whereby these actions occur are poorly understood. While changes in membrane fluidity would be expected to alter the function of a

variety of membrane-bound proteins, our results indicate that at least some of the physiologic changes that result from PUFAs cannot be explained only by the increase in membrane fluidity.

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