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Mechanism of apoptosis in HL-60 cells induced by n-3 and n-6 polyunsaturated fatty acids

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Abstract

The biochemical properties and specificity of n-3 and n-6 polyunsaturated fatty acids (PUFAs) are not well known. Because PUFAs induce apoptosis of different cells, we studied the effect of various PUFAs, such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosapentaenoic acid (DPA), on the fate of cultured human promyelocytic leukemia cells (HL-60) to elucidate the mechanism of apoptosis and the difference in action between n-3 and n-6 PUFAs. Fairly low concentrations of PUFAs inhibited the growth of HL-60 cells and induced their apoptosis by a mechanism that is sensitive to DMSO, an antioxidant, and z-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-fmk), a pan-caspase inhibitor. PUFAs stimulated the generation of reactive oxygen species (ROS) and activated various types of caspase-like proteases, such as caspase-3, -6, -8, and -9, but not caspase-1. In addition, PUFAs triggered the reaction leading to the cleavage of Bid, a death agonist member of the Bcl-2 family, and also released cytochrome *c* from mitochondria into the cytosol. PUFAs also decreased the mitochondrial membrane potential of intact HL-60 cells. All of these actions of n-3 PUFAs were stronger than those of AA, an n-6 PUFA, although the mechanism is not known. PUFAs stimulate swelling and membrane depolarization of isolated mitochondria in a cyclosporin A-sensitive manner. The results indicated that PUFA-induced apoptosis of HL-60 cells may be caused, in part, by direct action on the cells and by activation of the caspase cascade through cytochrome *c* release coupled with mitochondrial membrane depolarization.

Keywords: Apoptosis; Bid; Caspase; HL-60 cells; Membrane permeability transition; Polyunsaturated fatty acid

1. Introduction

PUFAs have been recognized as important energy sources and components of cell membranes. PUFAs also play key roles in many cellular events, such as the immune function [1], aging [2], neonatal development [3], anti-inflammatory effects [4], and gene regulation [5].

Dietary n-3 and n-6 PUFAs have potent biological effects on the blood (cells), the vasculature, and the myocardium. A great part of the PUFA effect can be explained by the known interference with eicosanoid metabolism [6]. It is well known that dietary n-3 and n-6 PUFAs have the ability to inhibit hepatic fatty acid biosynthesis. Central to this mechanism is the regulation of transcription of genes coding for lipogenic enzymes by dietary PUFAs. As regulators of gene expression, PUFAs (or metabolites) are thought to affect the activity of transcription factors [7]. Another interesting observation is the inhibitory effect of PUFAs on carnitine palmitoyltransferase I, which parallels the induction of apoptosis [8]. In this context, it is known that carnitine acts as a transporter of long chain fatty acids and

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Abbreviations: AA, arachidonic acid; CsA, cyclosporin A; Cyt.c, cytochrome *c;* DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; HE, hydroethidine; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide; MPT, membrane permeability transition; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; and z-VAD-fmk, z-Val-Ala-Asp(OMe)-fluoromethylketone.

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that acyl-L-carnitine suppresses apoptosis in several systems [9–11]. These findings suggested that fatty acid metabolism has an important role in cell growth and apoptosis.

Accumulated evidence has demonstrated that n-3 and n-6 PUFAs have similar actions on the biological functions of various cells [12,13]. However, dietary supplementation with n-3 PUFA resulted in a significant increase in the EPA content of neutrophils, a concomitant reduction in neutrophil AA, and a suppression of the generation of leukotriene B_4 in neutrophils [14]. Similarly, several investigators have shown that n-3 PUFAs have special biological effects, such as decreasing the AA content of membrane lipids [15], decreasing superoxide generation in neutrophils [16], decreasing neutrophil chemotaxis [17], inhibiting cancer cell growth [15], increasing lipid peroxidation [18], regulating vasorelaxation by a prostaglandin-dependent pathway [16], regulating gene expression [7], and inducing apoptosis [15, 19]. An examination of the effects of specific n-3 PUFAs demonstrated that the effect of EPA is related mainly to its inhibitory action on cell proliferation, whereas that of docosahexaenoic acid (DHA) corresponds with its induction of apoptosis [19]. In other words, the alterations in fatty acid composition in cells induced by n-3 PUFAs appear to be factors underlying their differential actions on cell proliferation and cell death by apoptosis. However, the molecular mechanisms of the different actions induced by n-3 and n-6 PUFAs remain obscure.

In these experiments, the effects of n-3 PUFAs on cell growth, differentiation, and apoptosis of HL-60 cells were examined in comparison with the effects of AA, an n-6 PUFA, to elucidate the molecular mechanism of PUFA-induced apoptosis and the specificity of n-3 PUFAs. The results of these experiments indicated that PUFA-induced apoptosis of HL-60 cells may be caused by activation of the caspase cascade coupled with the modulation of mitochondrial membrane functions.

2. Materials and methods

2.1. Chemicals

DCFH-DA, HE, JC-1, RNase A, and proteinase K were obtained from the Sigma Chemical Co. Anti-Cyt.c and anti-Bid antibodies were purchased from PharMingen and Santa Cruz Biotechnology, respectively. Acetyl-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA for caspase-3), acetyl-Ile-Glu-Thr-Asp-MCA (Ac-IETD-MCA for caspase-8), acetyl-Leu-Glu-His-Asp-MCA (Ac-LEHD-MCA for caspase-9), and the pan-caspase inhibitor z-VAD-fmk were obtained from the Peptide Institute. n-3 PUFAs were obtained from the Funakoshi Pharmaceutical Co. Ltd. All other chemicals were of analytical grade and obtained from Nacalai Tesque.

2.2. Preparation of PUFA–BSA complex

Fatty acids were presented to the cultured cells after being complexed with BSA in a molar ratio of 2.5:1 [13].

2.3. Cell line

HL-60 cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were grown in a humidified incubator at 37° under 5% CO_2/95% air and used for assays during the exponential phase of growth [20].

2.4. Subcellular fraction for Cyt.c assay

After harvesting, HL-60 cells ($\sim 10^7$) were suspended in 50 μL of ice-cold buffer A [250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and homogenized in a Teflon homogenizer. The homogenate was centrifuged at 750 *g* for 10 min at 4°. The supernatant was then centrifuged at 10,000 *g* for 15 min at 4°. The resulting pellet (mitochondrial fraction) was resuspended in buffer A. The supernatant was centrifuged further at 100,000 *g* for 60 min at 4°. The final supernatant represented the cytosolic fraction (cytosol). Aliquots of 20 μg were used for western blot analysis of Cyt.c [20].

2.5. Analysis of DNA fragmentation

The extent of DNA fragmentation was determined spectrophotometrically by the diphenylamine method, as described in detail previously [21]. DNA fragmentation was detected by agarose gel electrophoresis, as described previously [21].

2.6. Western blot analysis

Cell lysates ($\sim 10^7$) were prepared as described elsewhere [20]. The sample was diluted in SDS-sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue] and subjected to SDS–PAGE. Proteins in the gel were transferred onto an Immobilon filter (Millipore Co.), and then incubated with primary antibody (1:1000 dilution for Cyt.c, 1:200 dilution for Bid) and finally with a horseradish peroxidase-linked secondary antibody (1:2000 dilution for Cyt.c, 1:50,000 for Bid) and analyzed by using an ECL Plus kit (Amersham). Protein concentration was determined with a protein assay kit, using BSA as a standard.

2.7. Assay for caspase-like activity

The activities of caspase-like enzymes were determined as described previously [20,21] in 20 mM HEPES buffer

(pH 7.5) containing 0.1 M NaCl and 5 mM DTT at 37° for 1 hr using a 10 μM concentration of either Ac-DEVD-MCA, Ac-IETD-MCA, or Ac-LEHD-MCA for caspase-3, -8, and -9, respectively. The fluorescence of released 7-amino-4-methyl-coumarin (AMC) was measured using a fluorospectrophotometer. The wavelengths for excitation and emission were 355 and 460 nm, respectively.

2.8. Flow cytometric analysis of mitochondrial membrane potential in cells

HL-60 cells were washed twice with PBS, and stained with 2 μg/mL of JC-1 for 15 min at room temperature in the dark. After washing twice with PBS, cells were resuspended in PBS, and FACS analysis of JC-1 fluorescence was performed in a FACS Calibur flow cytometer (Becton Dickinson) to determine the mitochondrial membrane potential in the cells [22].

2.9. Flow cytometric analysis of intracellular generation of ROS

ROS in cells were measured by oxidation-sensitive fluorescent probes, DCFH-DA and HE [23]. Before and after incubation with various concentrations of PUFAs, cells were incubated for 30 min with 20 μM DCFH-DA or 10 μM HE in PBS containing 5 mM glucose, 0.3 mM $CaCl_2$, and 0.62 mM $MgCl_2$. Then the cells were washed with PBS and analyzed with a FACS Calibur flow cytometer.

2.10. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by the method of Hogeboom [24] using sucrose density gradient centrifugation, as described in a previous paper [25].

2.11. Assay for mitochondrial swelling and membrane potential

Mitochondria (100 μg protein/mL) were incubated in 10 mM Tris–HCl buffer (pH 7.4) containing 0.15 M KCl at 25°. Large amplitude swelling of mitochondria representing MPT [26] was measured spectrophotometrically at 540 nm using a dual beam spectrophotometer (Shimadzu UV-3000) equipped with a thermostatically controlled cuvette holder and a magnetic stirrer.

For the analysis of mitochondrial membrane potential, mitochondria (100 μg protein/mL) were incubated in the Tris–KCl solution at 25° in the presence of 0.1 μg/mL of cyanine dye, diS-C3-(5). Then the fluorescence intensity was recorded at 670 nm using excitation light at 622 nm in a fluorospectrophotometer (Hitachi 650–10LC) at 25° [27].

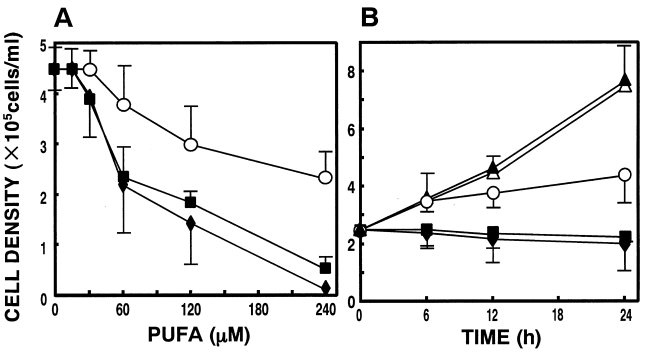

Fig. 1. Effect of various PUFAs on the growth of HL-60 cells. (A) HL-60 cells were exposed to various concentrations of PUFAs, such as AA (○), EPA (■), and DPA (♦) in the BSA-bound form as described in "Materials and methods." After 12 hr, the number of viable cells was determined by the trypan blue exclusion test. (B) Cells were incubated in the absence (Δ) or presence of BSA (▲) or 60 μM PUFAs for the indicated times. (A) and (B) show concentration- and time-dependent curves, respectively. Results are the means ± SD derived from three separate experiments.

3. Results

3.1. Effect of various PUFAs on cell growth and differentiation

The effect of various PUFAs on the growth of HL-60 cells was investigated. The presence of PUFAs inhibited the growth of HL-60 cells in a concentration- and time-dependent manner (Fig. 1, A and B). Cell growth was inhibited completely by BSA-bound PUFAs at concentrations higher than 60 and 120 μM, depending on the PUFA used. The inhibitory actions of PUFAs were in the order of DPA > EPA > AA. BSA had no appreciable effect on the growth of HL-60 cells.

3.2. Effect of PUFA on cellular DNA

Incubation of HL-60 cells with PUFAs induced the fragmentation of cellular DNA in a concentration- and time-dependent manner (Figs. 2 and 3). Kinetic analysis revealed that DNA fragmentation became apparent at 6–12 hr after incubation with 60–120 μM PUFAs. When treated with PUFAs at concentrations higher than 60 μM for 6 hr, a DNA ladder was observed on agarose gel electrophoresis. DNA ladder formation peaked 12 hr after treatment with 60 μM PUFAs. The ladder formation induced by both EPA and DPA was more pronounced than that induced by AA.

3.3. Effect of z-VAD-fmk on PUFA-induced DNA fragmentation

Because caspases play crucial roles in the process of apoptosis, we investigated the effect of z-VAD-fmk, a pan-caspase inhibitor [28,29], on EPA-induced apoptosis of HL-60 cells. DNA fragmentation induced by EPA was effectively suppressed by DMSO, a solvent for z-VAD-fmk,

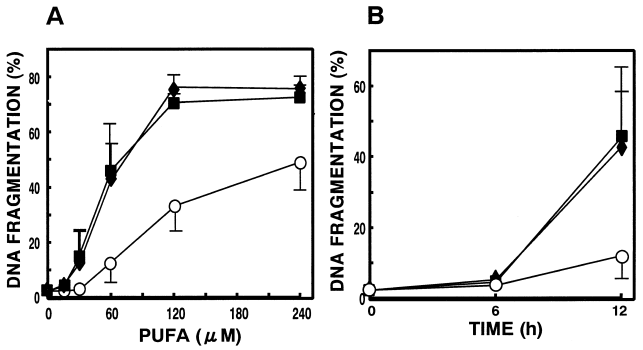

Fig. 2. DNA fragmentation of PUFA-treated HL-60 cells. Cells were incubated for 6 and 12 hr in the presence or absence of 60 μM PUFAs: AA (○), EPA (■), and DPA(♦). At the indicated time, fragmented DNA in the cells was determined by the diphenylamine method. (A) Concentration-dependent curves at 12 hr. (B) Time-dependent curves with 60 μM PUFAs. Results are means ± SD derived from three separate experiments.

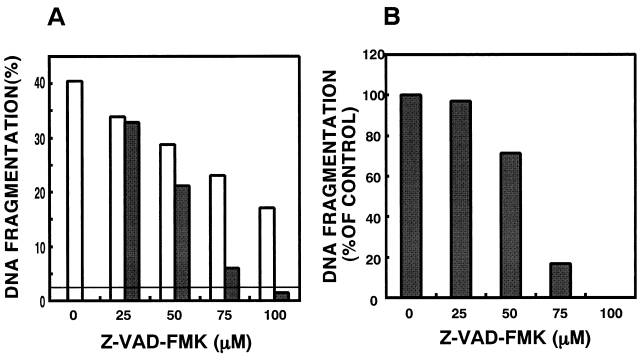

Fig. 4. Effect of z-VAD-fmk on the EPA-induced fragmentation of cellular DNA. Cells were preincubated in the presence or absence of 25–100 μM z-VAD-fmk dissolved in DMSO for 1 hr and then incubated with 60 μM EPA for 12 hr. DNA fragmentation was determined by the diphenylamine method. Key: (□) control, with DMSO and EPA; (■) EPA and 25, 50, 75, and 100 μM concentrations of added z-VAD-fmk dissolved in DMSO. Data are expressed as percent of DNA fragmentation (A) and percent of control (B).

when pretreating cells with 75 μM z-VAD-fmk for 1 hr (Fig. 4). Similar inhibition also was observed with the other PUFAs, i.e. AA and DPA (data not shown). The results indicate that EPA-induced DNA fragmentation is sensitive to DMSO, a scavenger of the hydroxyl radical, and that inactivation of caspases was also crucial for PUFA-induced apoptosis of HL-60 cells.

3.4. Effect of EPA on the generation of ROS by HL-60 cells

Since EPA-induced DNA fragmentation of HL-60 cells was inhibited by DMSO, it is possible that ROS might be generated in these cells. Thus, we studied the effect of EPA on the generation of ROS by HL-60 cells. Figure 5 shows the change in fluorescence intensity of DCFH, which is an oxidation-sensitive fluorescence probe [23]. When exposed to 60 μM EPA for various periods of time, the fluorescence intensity increased markedly, suggesting the generation of ROS (Fig. 5C, 6 hr). The generation of ROS depended upon the incubation time with EPA (Fig. 5B–D, 3–12 hr, respectively) and became apparent before the occurrence of cellular events leading to apoptosis. Similar results were obtained with other PUFAs (data not shown). These results indicate the involvement of ROS in the mechanism of PUFA-induced apoptosis.

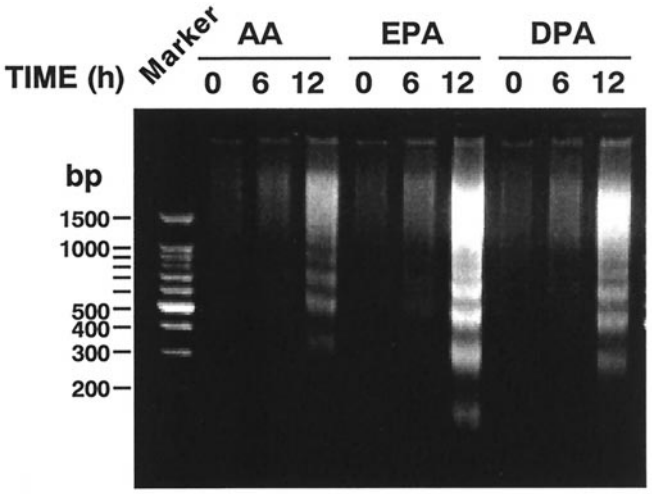

Fig. 3. DNA ladder formation of PUFA-treated HL-60 cells. Cells were incubated for 6 and 12 hr in the presence or absence of 60 μM PUFAs. At the indicated times, DNA was subjected to 2% agarose gel electrophoresis. The 100 bp ladder provides molecular size markers, and "TIME" (hr) is the incubation time with PUFAs. Similar results were obtained in three separate experiments.

3.5. Effect of PUFA on caspase-like activity

Because PUFA-induced apoptosis was inhibited by z-VAD-fmk, we also examined the effect of PUFAs on the stimulation of caspase-1-, -3-, -6-, -8-, and -9-like activities in HL-60 cells by using specific synthetic substrates for each caspase. PUFAs activated various types of caspase-like proteases, such as caspase-3, -6, -8, and -9, but not caspase-1. The cellular activities of the caspases increased after incubation for 6 hr with a variety of PUFAs (60 μM) and reached a maximum at 12 hr (Fig. 6) (data for caspase 1 and 6 are not shown). The PUFA-induced stimulation of caspase-3- and -6-like activities in the cells was more pronounced than stimulation of caspase-8- and -9-like activities. The results obtained in these experiments suggest that a cascade of caspase proteases is involved in the PUFA-induced apoptosis of HL-60 cells.

3.6. PUFA-induced release of Cyt.c from mitochondria

Because PUFAs activated caspase-9, it was surmised that Cyt.c was being released from the mitochondria into the cytosol [30]. As suspected, a significant fraction of the Cyt.c was released from the mitochondria of PUFA-treated cells

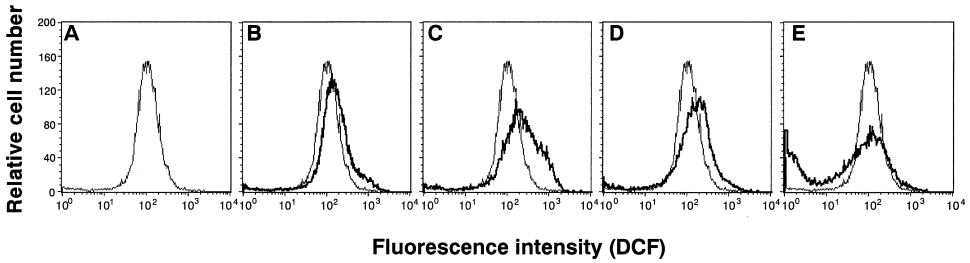

Fig. 5. Stimulation by EPA of ROS generation in HL-60 cells. ROS were measured using an oxidation-sensitive fluorescent probe, DCFH-DA. Cells were treated with 60 μM EPA for 3, 6, 12, and 24 hr, and then incubated for 30 min with 20 μM DCFH-DA at 37°. After washing with PBS, the fluorescence intensity of the cells was analyzed with a FACS Calibur flow cytometer. Similar results were obtained in three separate experiments. (A) Control without treatment; (B) 3 hr; (C) 6 hr; (D) 12 hr; and (E) 24 hr.

(Fig. 7). This Cyt.c release was partially inhibited by z-VAD-fmk. The amounts of Cyt.c released by EPA and DPA were higher than that released by AA. This result indicated that the release of Cyt.c from mitochondria might play an important role in PUFA-induced apoptosis of HL-60 cells.

3.7. Effect of PUFA on the cleavage of cellular Bid

Bid cleaved by caspase-8 directly triggers the release of Cyt.c from mitochondria [31–33] (without swelling and depolarization of the inner membrane) by some Bcl-2-inhibitable mechanism [32], thus relaying an apoptotic signal from the cell surface to the mitochondria [31]. Because of this observation, we studied the effect of PUFA on the cellular levels of cleaved Bid. Western blot analysis revealed that Bid was present as a 22-kDa protein in intact HL-60 cells. Incubation of cells with PUFAs resulted in the formation of 13- and 15-kDa fragments of Bid (Fig. 8). The cleavage of Bid was inhibited by the presence of z-VAD-fmk (data not shown). The ability of PUFAs to stimulate the cleavage of Bid was more pronounced in the case of EPA and DPA than in the case of AA. These results showed that the cleaved products of Bid were involved in the PUFA-induced release of Cyt.c.

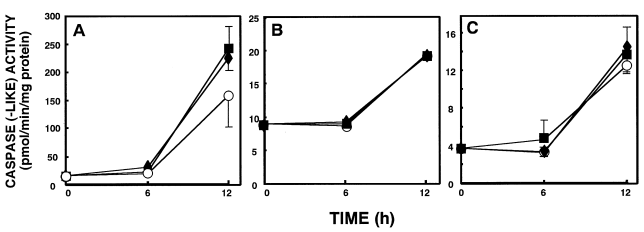

Fig. 6. Activation of various caspase (-like) enzymes of HL-60 cells by PUFAs. Cells were incubated with PUFAs (60 μM), and time-dependent changes in the activities of caspase-3 (A), -8 (B), and -9 (C) (-like) enzymes were determined as described in the text. Cell extracts were incubated with 10 μM fluorogenic peptide substrates at 37° for 1 hr. Results are the means ± SD derived from three separate experiments. Key: (○) AA; (■) EPA; and (♦) DPA.

3.8. EPA-induced decrease in mitochondrial membrane potential in HL-60 cells

It has been shown that Cyt.c was released from mitochondria into the cytosol by opening of the pore during MPT, and changes in the opening of this pore have been postulated to play a role in cellular events leading to apoptosis of certain types of cells [26,30]. For these reasons, the effect of PUFAs on the membrane potential of intracellular mitochondria in intact cells was examined using FACS analysis of JC-1 fluorescence. After treatment of HL-60 cells with 60 μM EPA for 6, 12, and 24 hr, the intensity of the JC-1 fluorescence was decreased in a time-dependent manner concomitant with DNA fragmentation (Fig. 9). This EPA-induced depolarization of the mitochondrial membrane potential in HL-60 cells was partially suppressed in the presence of z-VAD-fmk (data not shown). Similar results were obtained by treatment with other PUFAs (data not shown). The EPA-induced depolarization of the membrane potential was greater than that induced by AA. These results indicate that mitochondrial MPT is involved in the apoptosis induced by treatment with PUFA.

3.9. Effect of EPA on the swelling, membrane potential, and Cyt.c release of isolated mitochondria

Because EPA induced the depolarization of the mitochondrial membrane potential in HL-60 cells and released Cyt.c, the effect of free EPA on the function of isolated mitochondria was examined. EPA depolarized the mem-

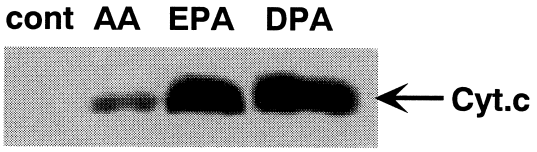

Fig. 7. PUFA-induced release of Cyt.c from mitochondria in HL-60 cells. Cells were treated with 60 μM PUFAs for 12 hr after which the amount of Cyt.c in the cytosolic fraction was determined by western blotting. Molecular mass of Cyt.c: 15 kDa. Similar results were obtained in three separate experiments.

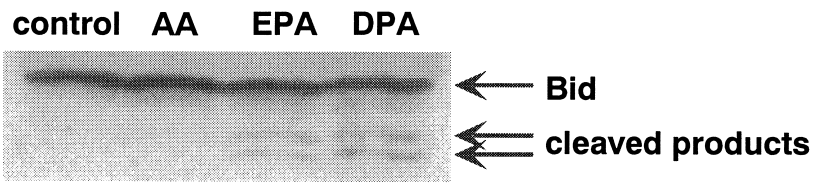

Fig. 8. Increase in cellular levels of cleavage products of Bid induced by PUFAs. Cells were incubated in the presence or absence of 60 μM PUFAs for 12 hr. Cell lysates (30 μg) were analyzed by immunoblotting. Molecular masses: 22 kDa, pro-Bid; 13 and 15 kDa, processed Bid. Similar results were obtained in three separate experiments.

brane potential of isolated mitochondria in a CsA-sensitive manner (Fig. 10). Large amplitude swelling of mitochondria and Cyt.c release were also induced by EPA in the presence of low concentrations of Ca^{2+} in a CsA-sensitive manner. The concentration of EPA required to induce mitochondrial swelling was less than 10 μM. These effects were also observed with free AA and DPA. However, the concentration of DPA required to induce MPT was lower than those of AA and EPA (data not shown). Furthermore, BSA-bound PUFAs failed to induce mitochondrial MPT at the same concentration as that required to induce apoptosis. These results indicate that PUFAs may not directly trigger the release of Cyt.c from mitochondria in the cells.

4. Discussion

A wide variety of metabolites of fatty acids will trigger the induction of MPT in the mitochondrial membrane [34]. Although we showed in this study that various PUFAs induced apoptosis of HL-60 cells, it is well known that these cells are notoriously sensitive to induction of apoptosis. However, a similar apoptosis induced by EPA was also observed in PC12 neuronal cells and HepG2 hepatoma cells (data not shown). A possible molecular mechanism of this process involves the interaction of fatty acid metabolites with the adenine nucleotide translocase of the inner membrane, thereby forming an additional route for dissipation of the proton gradient [35–37]. The present work clearly demonstrates that PUFAs inhibited the growth of HL-60 cells and also induced apoptosis through modulation of mitochondrial functions, thereby releasing Cyt.c from the mitochondria into the cytosol. The released Cyt.c activated caspase-3 through formation of an apoptotic protease activating factor (Apaf) complex in the cytosol [30] and induced apoptosis of HL-60 cells without triggering their differentiation to granulocytes. In this case, Cyt.c release has been postulated to be the result of depolarization of the mitochondrial membrane and the opening of the pore during MPT [30]. However, kinetic analysis revealed that an increase in the generation of caspase-8-cleaved Bid enhanced the release of Cyt.c from the mitochondria into the cytosol in a manner that was independent of mitochondrial membrane depolarization [32,33]. These results indicate that there are at least two mechanisms for the release of Cyt.c from the mitochondria into the cytosol. One depends upon membrane depolarization, but the other does not. To analyze the mechanism, we studied the effects of PUFAs on cellular events occurring in HL-60 cells, such as the depolarization of the mitochondrial membrane potential of cells by use of JC-1 [22] and cleavage of Bid in the cytoplasm. We confirmed that the mitochondrial membrane potential was depolarized at 6 hr after treatment with PUFAs and that caspase-8 was activated and Bid cleaved. These observa-

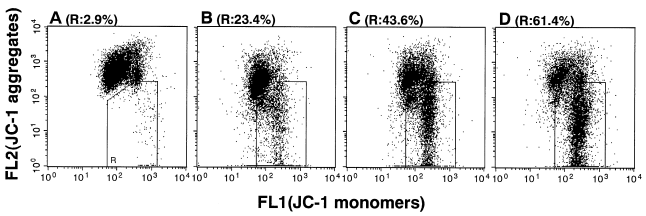

Fig. 9. EPA-induced depolarization of the mitochondrial membrane potential in HL-60 cells. After treatment of HL-60 cells with 60 μM EPA for 6, 12, and 24 hr, cells were washed twice with PBS and stained with 2 ng/mL of JC-1. The intensity of JC-1 fluorescence was analyzed by a FACS Calibur flow cytometer to determine the mitochondrial membrane potential in the cells. Similar results were obtained in three separate experiments. (A) Control without treatment; (B) 6 hr; (C) 12 hr; and (D) 24 hr.

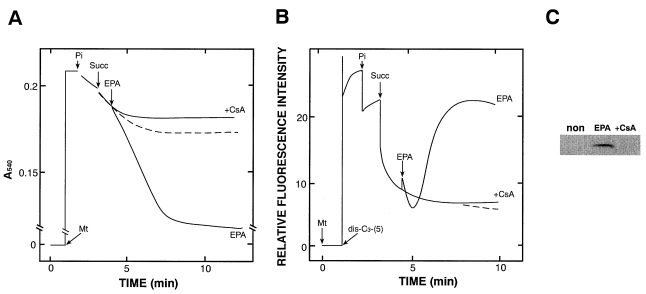

Fig. 10. Induction of mitochondrial MPT and Cyt.c release by EPA. Rat liver mitochondria (0.1 mg protein/mL) were incubated in 0.15 M KCl containing 10 mM Tris–HCl buffer (pH 7.4) at 25°. The mitochondrial membrane potential and swelling were measured by the fluorescence change of diS-C3-(5) and the absorbancy at 540 nm, respectively. Cyt.c release after a 10-min treatment with 10 μM EPA in the presence or absence of CsA was detected by western blot analysis. Concentrations of added diS-C3-(5), mitochondria, P_i, succinate, and EPA were 0.1 μg/mL, 0.1 mg protein/mL, 2 mM, 5 mM, and 10 μM, respectively. CsA (1 μM) was added before the incubation of the mitochondria. Similar results were obtained in three separate experiments. (A) Swelling. (B) Membrane depolarization. (C) Cyt.c release.

tions indicated that Cyt.c release in HL-60 cells occurred through both depolarization-dependent and -independent mechanisms. In this context, it is possible that membrane depolarization might be due to the results of the direct action of PUFAs on the mitochondrial membrane. To test this possibility, the effect of PUFAs on the membrane potential of isolated mitochondria was examined, and it was found that PUFAs induced the MPT, which corresponded to the membrane depolarization and swelling of mitochondria observed. Thus, it appears that the release of Cyt.c might be a part of the cause of PUFA-induced apoptosis of HL-60 cells. During the preparation of this manuscript, we came across a paper that reported that saturated long chain fatty acids, such as palmitate, also induced the apoptosis of cardiomyocytes through modification of mitochondrial functions [38]. In this context, certain specific structural features must be present in the PUFA molecule to induce apoptosis of HL-60 cells. Induction depends on the number of double bonds and the chain length of the fatty acids. Furthermore, higher concentrations of saturated fatty acids, such as 100 μM palmitic acid, also induced apoptosis of the cells. Moreover, it was found that some of the AA metabolites, such as 15-HPETE or 12(*S*)-HETE, induced apoptosis of Y79 cells [39] or protected tumor cells from apoptosis [40], respectively. Thus, further studies are required to clarify the structural specificity.

Another cause of PUFA-induced apoptosis is ROS. It is well known that PUFAs stimulate the generation of ROS [41]. The generation of ROS in HL-60 cells was detected by an oxidation-sensitive fluorescent probe, DCFH-DA or HE. In this case, ROS reacting with DCFH are thought to be essentially specific to hydrogen peroxide rather than to the superoxide radical. Furthermore, DMSO inhibited PUFA-induced DNA fragmentation. These results indicate that the original species might be free radicals and generated hydroxyl radicals. However, the molecular mechanism of apoptosis induced by ROS and the regulation of the mechanism of caspase activation by ROS are unclear. In this context, we found that all of these actions of n-3 PUFAs were stronger than those of AA. It has been reported recently that conjugated EPA and DHA induced apoptosis via lipid peroxidation in cultured human tumor cells [42]. In preliminary experiments in this laboratory, we observed that the values of TBA-reactive substances (TBARS) in BSA-EPA or BSA-DPA and in cells treated with BSA-EPA and BSA-DPA were higher than those of AA. The mechanism of apoptotic cell death induced by these peroxidized lipids is currently being investigated.

Another possible explanation for the cytotoxicity of PUFAs would be the surfactant nature of the fatty acid molecules. Because PUFAs interact with cellular membranes, it might directly affect the stability and functions of these membranes and modulate membrane-bound enzymes and/or phospholipids, thereby initiating a sequence of events leading to apoptosis. This possibility should also be studied further.

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