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Involvement of hydroperoxide in mitochondria in the induction of apoptosis by the eicosapentaenoic acid

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Abstract
Eicosapentaenoic acid (EPA) induced apoptosis of rat basophilic leukemia cells (RBL2H3 cells), whereas 100 μM linoleic acid (LA) had no significant effect. Cytochrome *c* was released at 4 h. Apoptosis was detected at 6 h after exposure to EPA and docosahexaenoic acid (DHA), and preceded the activation of caspase-3. Liberation of apoptosis-inducing factor (AIF) from mitochondria and its translocation into the nucleus were observed at 4 h. A broad-specificity caspase inhibitor, z-VAD-fmk, failed to suppress the apoptosis, suggesting that EPA induced caspase-independent apoptosis. On other hand, a poly(ADP-ribose) polymerase-1 (PARP-1) inhibitor that blocks AIF translocation to the nucleus suppressed EPA-induced apoptosis. The level of hydroperoxide in the cells and mitochondria increased at the early phase of apoptosis within 2 h. On the contrary, elevation of hydroperoxide in mitochondria was not observed after treatment with LA. The EPA-induced apoptosis was abolished by prevention of the hydroperoxide elevation in mitochondria via overexpression of mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPx). Neither cytochrome *c* nor AIF were released from mitochondria in the mitochondrial PHGPx-overexpressing cells. EPA also induced apoptosis in HeLa cells, but not in L929 or RAW264.7 cells. Enhancement of the hydroperoxide level in mitochondria was found in the EPA-sensitive HeLa cells after treatment with EPA, whereas no such enhancement was observed in the apoptosis-resistant L929 and RAW264.7 cells. These results suggest that the generation of hydroperoxide in mitochondria induced by EPA is associated with AIF release from mitochondria and the induction of apoptosis.

Keywords: *Eicosapentaenoic acid, apoptosis, mitochondria, phospholipid hydroperoxide glutathione peroxidase, hydroperoxide, apoptosis-inducing factor*

Abbreviations: *PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; PARP-1, poly(ADP-ribose) polymerase-1; AIF, apoptosis-inducing factor; PHGPx, phospholipid hydroperoxide glutathione peroxidase; ROS, reactive oxygen species; z-VAD-fmk, z-Val-Ala-Asp-(OMe)-fluoromethylketone; Ac-DEVD-MCA, acetyl-DEVD-4-methyl-cumaryl-7-amide; DHIQ, 1,5-dihydroxyisoquinoline; DHR, dihydrorhodamine 123; DHE, dihydroethidium; ANT, adenine nucleotide translocator; PT pore, permeability transition pore*

Introduction

Polyunsaturated fatty acids (PUFAs) are fatty acids with more than 12 carbons and 2 or more double bonds in their carbon chains. The hydrocarbon chain of eicosapentaenoic acid (EPA) comprises 20 carbon atoms and 5 double bonds. EPA is a member of *n*-3 family of fatty acids. During the past few decades, dietary PUFAs have been suggested as a potential group of natural products that play significant roles in modulating cancer development [1]. Epidemiological studies have indicated a relationship between high fat consumption and colorectal cancer [2]. The low colon cancer rate among Alaskan and Greenland Eskimos is associated with their high consumption of large amounts of fish that contain *n*-3 PUFAs, mainly EPA and docosahexaenoic acid (DHA) [3,4]. Diets rich in *n*-3 family PUFAs have been linked to a reduced

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risk of cancer development [5]. In animal models, dietary supplementation with fish oil inhibits chemically-induced colon carcinogenesis [6,7] and increases apoptosis [8] in rats. The effects of PUFAs on cell growth have been investigated using a variety of cultured tumor cell lines [9–12]. Recent studies have indicated that PUFAs, including EPA, play an important role in the regulatory mechanism controlling cellular proliferation and apoptosis [13–15]. PUFAs induced cell death in various kinds of cancer cells that showed characteristic apoptotic changes, such as phosphatidylserine externalization, chromatin condensation and caspase activation [15,16]. Repression of apoptosis is one of the main underlying problems in cancer development, and disruption of the apoptotic program can promote tumor initiation, progression and resistance to treatment [17]. However, far less information is available with respect to the mechanism of action of PUFAs, in terms of their effect on the induction of apoptosis, although the inductive effect of EPA on apoptosis has been described [18–22].

Reactive oxygen species (ROS) are toxic by-products of PUFA degradation. It has recently been revealed that ROS modulate the physical state of cells and influence cell death [23]. A relationship between ROS and apoptosis has been suggested by many experimental observations. Apoptosis is induced by pro-oxidant agents, such as hydrogen peroxide, diamide, etoposide and semiquinones [24,25]. In addition to the production of ROS from PUFAs, oxidized metabolites produced by the peroxidation of PUFAs induce apoptosis. Arita et al. demonstrated that the oxidized products of *n*-3 or *n*-6 PUFAs produced by ultraviolet irradiation induced apoptosis in HL-60 cells [26]. It has been reported that oxidized *n*-3 PUFAs, such as conjugated EPA and conjugated DHA, exhibit cytotoxicity in cancer cells by inducing apoptosis through lipid peroxidation [27]. Antioxidants, such as *N*-acetylcysteine, suppress the apoptosis by acting as ROS scavengers, providing additional evidence that ROS may act as signaling molecules to initiate apoptosis [28]. These previous reports suggest that ROS are involved in the initiation of apoptosis induced by PUFAs through peroxidation. However, little is known about the role of ROS in PUFA-induced apoptosis.

In this study, we demonstrate that EPA causes elevation of hydroperoxide in mitochondria and induces the release of proapoptotic factors, such as cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria. The apoptosis induced by EPA was potently suppressed by overexpression of mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPx) through scavenging of the hydroperoxide generated in mitochondria. These results suggest that the hydroperoxide produced in mitochondria may play a crucial role in the initiation of apoptosis by EPA.

Material and methods

Reagents

Dihydrorhodamine 123 (DHR) and dihydroethidium (DHE) were obtained from Molecular Probes Inc. (Leiden, The Netherlands). RNase was purchased from Roche Molecular Biochemicals (Almere, The Netherlands). The anti-cytochrome *c* antibody was from PharMingen Inc. (San Diego, CA). The anti-AIF antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). HRP-conjugated rabbit anti-mouse IgG was purchased from Zymed Lab. (San Diego, CA). Ac-DEVD-MCA was purchased from Peptide Institute Inc. (Osaka, Japan). Free fatty acids and z-VAD-fmk were obtained from Funakoshi Pharmaceutical Co. Ltd. (Tokyo, Japan). DMEM was from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Block Ace was from Dainippon Pharmaceutical Co. (Osaka, Japan). All other chemicals, including DHIQ, were obtained from WAKO Pure Chemicals Inc. Ltd. (Tokyo, Japan).

Cell culture

RBL2H3, HeLa, L929 and RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS). Cell were grown in a humidified incubator at 37°C under 5% CO_2/95% air and used for assays during the exponential phase of growth. We also used a previously established strain of RBL-2H3 cells in which PHGPx was overexpressed in the mitochondria [29].

Assessment of cell viability

Cells were plated at 1×10^4 cells/well in flat-bottomed 96-well culture plates and cultured in DMEM containing 5% FCS for 24 h. Cells were washed with phosphate-buffered saline (PBS, pH 7.4), suspended in 100 μl of DMEM containing free fatty acids which was dissolved in ethanol and incubated for the appropriate period in a humidified incubator at 37°C. The cell viability was then determined by measuring the release of lactate dehydrogenase (LDH) as described previously [29].

DNA fragmentation

At the indicated times, 2×10^6 cells were centrifuged into a pellet and then resuspended in 250 μl of Tris-EDTA buffer (1 mM EDTA, 10 mM Tris–HCl, pH 7.6). An equal amount of ice-cold lysis buffer (0.5% Triton X-100, 2 mM EDTA, 5 mM Tris–HCl, pH 8.0) was then added, and the cells were lysed for

30 min before centrifugation at 12,000 rpm for 20 min. The DNA in the supernatant was precipitated with ethanol, treated with RNase at 37°C for 30 min, and then extracted with phenol and chloroform. The recovered DNA fragments were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Measurement of caspase-3 activity

Cells (10^7 cells) were treated with 50 μM EPA for the indicated times, then washed twice with PBS and incubated in 400 μl of PBS containing 10 μg/ml digitonin at 37°C for 5 min. The lysate was collected and centrifuged at 12,000 rpm for 20 min. The supernatant was diluted with 400 μl of reaction buffer (1 mM dithiothreitol, 2 mM EDTA, 0.1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 mM Tris–HCl, pH 7.4) and then incubated with 50 μmol of Ac-DEVD-MCA, as the substrate for caspase-3, at 37°C for 30 min. The fluorescence of 7-amino-4-methylcoumarin cleaved from Ac-DEVD-MCA by caspase-3 was measured using a CytoFluor system (model 4000; PerSeptive Biosystems, Framingham, MA) with excitation at 380 nm and emission at 460 nm.

Release of cytochrome c

Cells (10^7 cells) were treated with 50 μM of EPA, and the supernatants of the cell lysates after digitonin treatment were collected as described above to analyze the release of cytochrome *c* from mitochondria into the cytosol. The proteins in the supernatant were precipitated by addition of trichloroacetic acid. The precipitated proteins and mitochondrial pellet were suspended in 30 μl of sample buffer (10% glycerol, 1% SDS, 60 mM Tris–HCl, pH 6.8) and then fractionated by SDS-PAGE (15% polyacrylamide) under non-reducing conditions. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Belford, MA) as described previously [3–26]. The PVDF membrane was blocked by incubation with Block Ace for 1 h and then incubated with an anti-cytochrome *c* antibody for 2 h. The PVDF membrane was subsequently incubated for 1 h with HRP-conjugated anti-mouse IgG antibodies. The antibody binding to the PVDF membrane was detected using an enhanced chemiluminescence western blotting analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Redistribution of AIF

Cells (2×10^7 cells) were treated with 50 μM EPA for 4 h, and then the cell nuclei, mitochondria and cytosol were sub-fractionated by differential centrifugation of a cell homogenate as described previously [29]. The proteins in the supernatant were precipitated by addition of trichloroacetic acid. The precipitated proteins, mitochondrial pellet and nuclear pellet were suspended in sample buffer and fractionated by SDS-PAGE (12.5% polyacrylamide) under non-reducing conditions. The proteins in the subcellular fractions were detected as described above.

Measurement of hydroperoxide and superoxide generation

The intracellular hydroperoxide and superoxide levels were monitored by measuring the changes in fluorescence that resulted from oxidation of an intracellular probe. To assess the levels of intracellular hydroperoxide, we used 2 μg/ml DHR, which is oxidized by hydrogen peroxide and lipid hydroperoxide to yield fluorescent rhodamine 123. To assess the levels of superoxide, we used 2 μg/ml DHE, which is oxidized by superoxide to yield fluorescent ethidium. Cells were plated at 10^5 cells/well in a 12-well plate, washed three times with PBS, and then incubated in DMEM containing DHR or DHE. EPA was added to give a final concentration of 50 μM in a total volume of 1 ml. The fluorescence was monitored using a CytoFluor plate-reader.

To assess the levels of mitochondrial hydroperoxide, isolated mitochondria treated with or without EPA for 4 h were prepared by differential centrifugation of a cell homogenate as described previously [29]. Aliquots of the mitochondria were incubated with 2 μg/ml DHR for 20 min and the fluorescence was monitored using a CytoFluor plate-reader.

Protein quantification

The protein concentrations were determined using the BCA protein assay reagent (Pierce) with bovine serum albumin as the standard.

Results

Induction of apoptosis by PUFAs

Cells were cultured in the presence of PUFAs and monitored in terms of the LDH release (Figure 1). The numbers of dead cells increased gradually after incubation with 50 μM EPA or DHA for 6 h and reached 60% of the total cells at 18 h. The cytotoxic effects of EPA and DHA on RBL2H3 cells were both dose- and time-dependent. Arachidonic acid (AA) had less effect on the cell death than EPA and DHA, while 100 μM linoleic acid (LA) did not induce cell death.

Cleavage of DNA into a "ladder" of fragments was detected after incubation with EPA and DHA for 18 h (Figure 2). The ladder formation induced by both EPA and DHA was more pronounced than that induced by AA, while DNA fragmentation was not

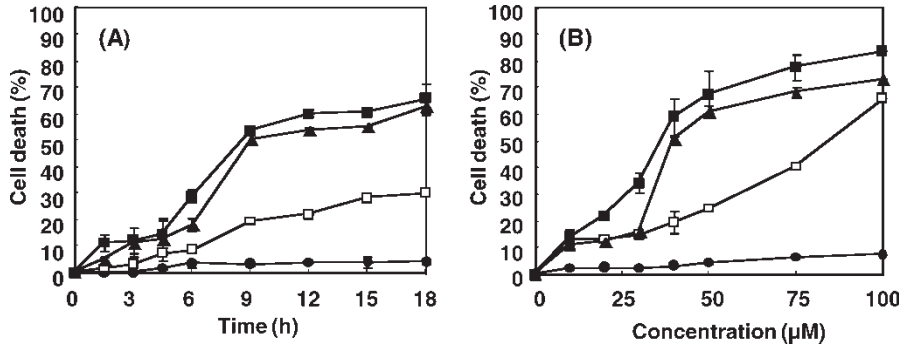

Figure 1. Effects of various fatty acids on the cell death of RBL2H3 cells. RBL2H3 cells were exposed to fatty acids, including LA (closed circles), AA (open squares), EPA (closed triangles) and DHA (closed squares). (A) Cells were incubated with 50 μM of each fatty acid for the indicated times. (B) Cells were exposed to the indicated dose of each fatty acid for 18 h. The cell viability was estimated from the release of LDH. Data are the means ± SD of three replicates in each case.

observed after incubation with LA. These results clearly show that EPA and DHA induced apoptotic cell death in RBL2H3 cells.

Cytochrome c release and caspase-3 activation

The release of cytochrome *c* from mitochondria was monitored in order to examine the signaling pathway of EPA-induced apoptosis. Cytochrome *c* was detectable in cells at 4 h after exposure to EPA (Figure 3A). The proteolytic activity of caspase was measured by the cleavage of Ac-DEVD-MCA, a substrate for caspase-3. Significant caspase-3 activation was not detected at 6 h, although apoptosis was initiated at this time. Activation of caspase-3 was evident at 18 h after exposure to EPA (Figure 3B).

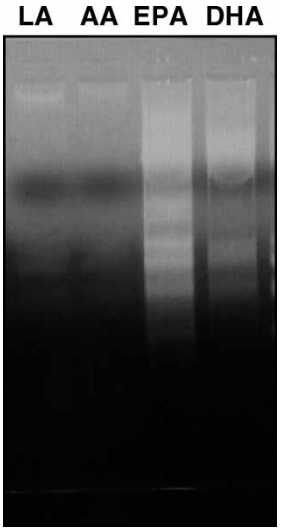

Figure 2. DNA fragmentation of RBL2H3 cells during exposure to PUFAs. Cells were treated with 50 μM of each fatty acid for 18 h. Low molecular weight DNA was recovered from the fatty acid-treated cells, fractionated by gel electrophoresis and then stained with ethidium bromide.

The z-VAD-fmk (up to 500 μM) which is a broad-specificity caspase inhibitor did not protect against the apoptosis caused by EPA, suggesting that caspase activation was not associated with the process of apoptosis and that EPA induced apoptosis via a caspase-3-independent pathway (data not shown).

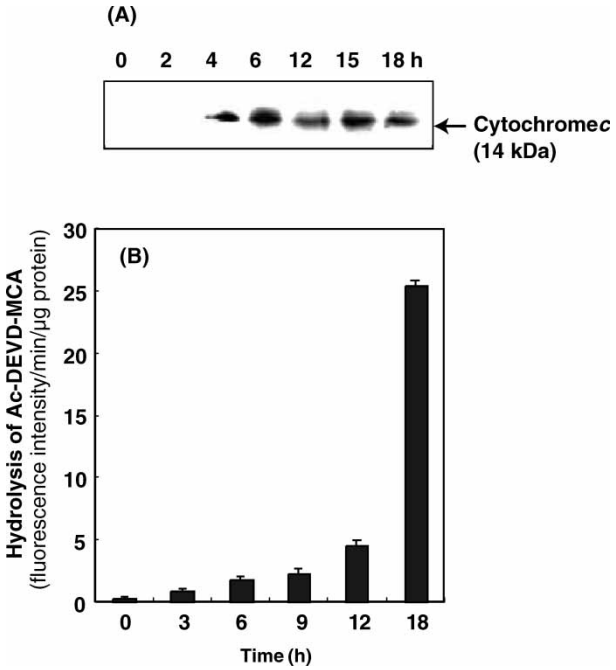

Figure 3. Cytochrome *c* release and caspase-3 activation during the apoptosis induced by EPA. RBL2H3 cells were treated with 50 μM EPA for the indicated times. The cytosolic fractions were then prepared after treatment with digitonin for 10 min. (A) Cytochrome *c* release was detected by immunoblotting analysis with an anti-cytochrome *c* antibody. (B) Caspase-3-like proteolytic activity was determined by monitoring the cleavage of the fluorogenic substrate Ac-DEVD-MCA. The activity in the cytosol is represented as the fluorescence intensity of 7-amino-4-methylcoumarin/min/μg protein. Data are the means ± SD of four replicates in each case.

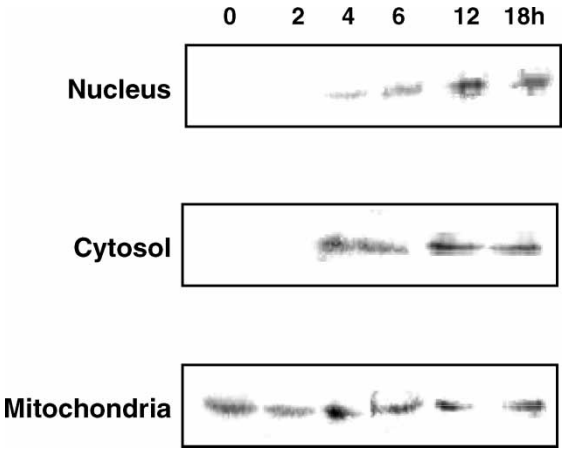

Figure 4. Translocation of AIF from mitochondria to the nucleus during the apoptosis induced by EPA. RBL2H3 cells were treated with 50 μM EPA for the indicated times. Cytosolic and nuclear fractions were then prepared and the release of AIF was detected by immunoblotting with an anti-AIF antibody.

Translocation of AIF and effect of a poly(ADP-ribose) polymerase-1 inhibitor on apoptosis

AIF is released from mitochondria and translocated into the nucleus in response to apoptotic stimuli, where it causes peripheral chromatin condensation and large scale DNA fragmentation in a caspase-independent manner. EPA induced the release of AIF at 4 h and its translocation to the nucleus (Figure 4). Pretreatment with 1,5-dihydroxyisoquinoline (DHIQ), a specific inhibitor of poly(ADP-ribose) polymerase-1 (PARP-1) activity [30], effectively inhibited the apoptosis induced by EPA (Figure 5). DHIQ did not influence the release of cytochrome *c* into the cytosol, but did disrupt AIF translocation from mitochondria to the nucleus, indicating that AIF translocation into nucleus is associated with the process of the apoptosis induced by EPA (Figure 6).

Production of superoxide and hydroperoxide in response to EPA

The levels of superoxide and hydroperoxide were determined using specific fluorescent indicators (Figure 7A,B). Marked increases in the levels of intracellular superoxide and hydroperoxide, including hydrogen and lipid peroxide, were observed after cell exposure to EPA. Intracellular superoxide was detectable in cells within 2 h of exposure to EPA, and the level increased at a steady rate up to 4 h. Intracellular hydroperoxide also produced at 2 h after EPA addition and increased linearly up to 8 h. The levels of hydroperoxide were determined in mitochondria isolated from RBL2H3 cells treated with or without EPA (Figure 7C). A low level of hydroperoxide was detected in mitochondria without EPA treatment, whereas the level was markedly increased in mitochondria after treatment with EPA for 4 h. In

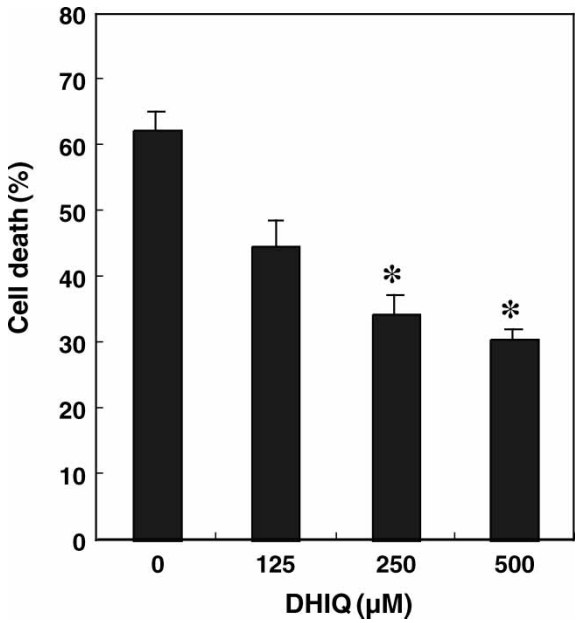

Figure 5. Effect of DHIQ on the apoptosis induced by EPA. RBL2H3 cells were preincubated in the presence or absence of DHIQ (125–500 μM) for 3 h and then incubated with 50 μM EPA for 18 h. The cell viability was estimated from the release of LDH. Data are the means ± S.D of three replicates in each case. $^{*}p < 0.05$ in comparison to unstimulated control.

contrast, no significant elevation of hydroperoxide was observed in mitochondria after treatment with LA, which failed to induce apoptosis.

Effect of EPA on the viability of HeLa, L929 and RAW264.7 cells and the production of hydroperoxide in mitochondria

The effects of EPA on the viability of other kinds of cell lines were determined (Figure 8). Similarly to RBL2H3 cells, HeLa cells were sensitive to treatments

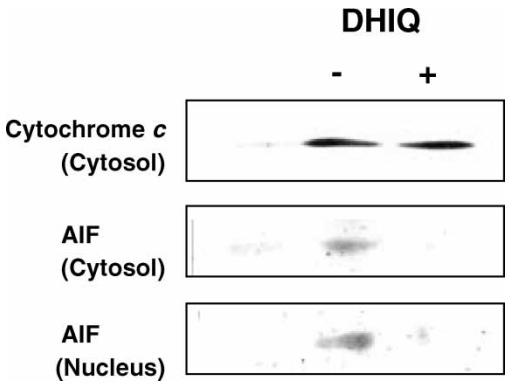

Figure 6. Effect of DHIQ on the release of cytochrome *c* and AIF induced by EPA. RBL2H3 cells were preincubated in the presence or absence of DHIQ (125 μM) for 3 h and then incubated with 50 μM EPA for 6 h. Cytosolic and nuclear fractions were then prepared and the release of AIF or cytochrome *c* was detected by immunoblotting with an anti-AIF or anti-cytochrome *c* antibody, respectively.

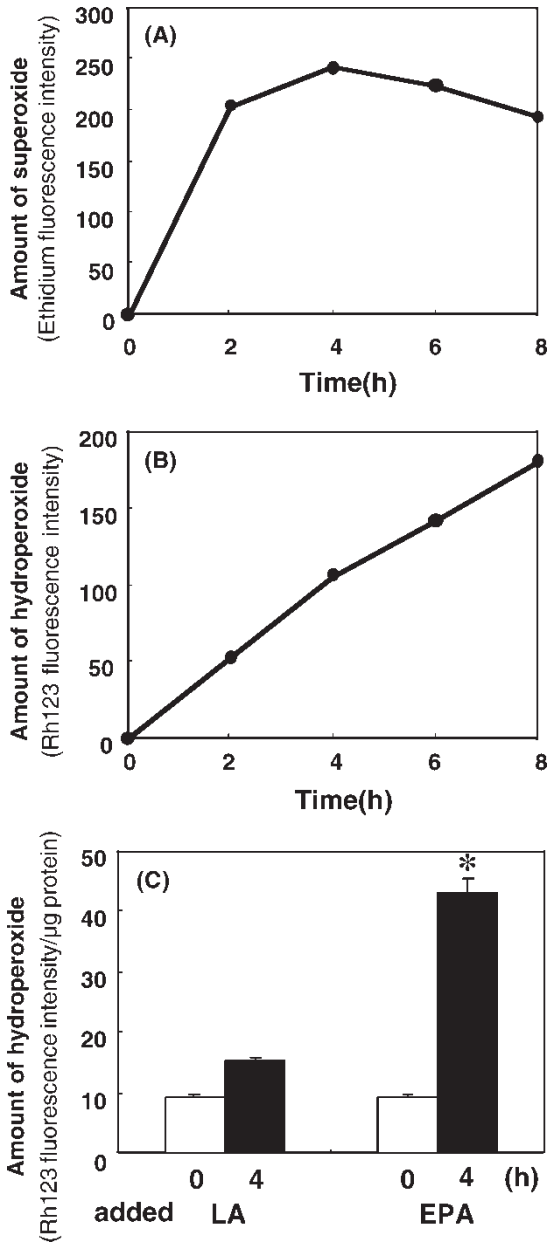

Figure 7. Intracellular and mitochondrial levels of superoxide and hydroperoxide in RBL2H3 cells treated with EPA. Cells were incubated in medium containing DHE (A) or DHR (B) in the presence or absence of 50 μM EPA. After incubation for the indicated times, the fluorescence intensities of ethidium and rhodamine 123 (Rh123) in the cells were quantified using a CytoFluor plate-reader. The fluorescence intensities in EPA-treated cells are expressed relative to the intensities in untreated cells. Each point represents the average of triplicate results. (C) Mitochondria were isolated from cells treated with (closed bars) or without (open bars) EPA for 4 h. The isolated mitochondria were incubated with DHR and the fluorescence intensity was measured. Data are the means ± SD of three replicates in each case. $*P < 0.05$ was considered significant.

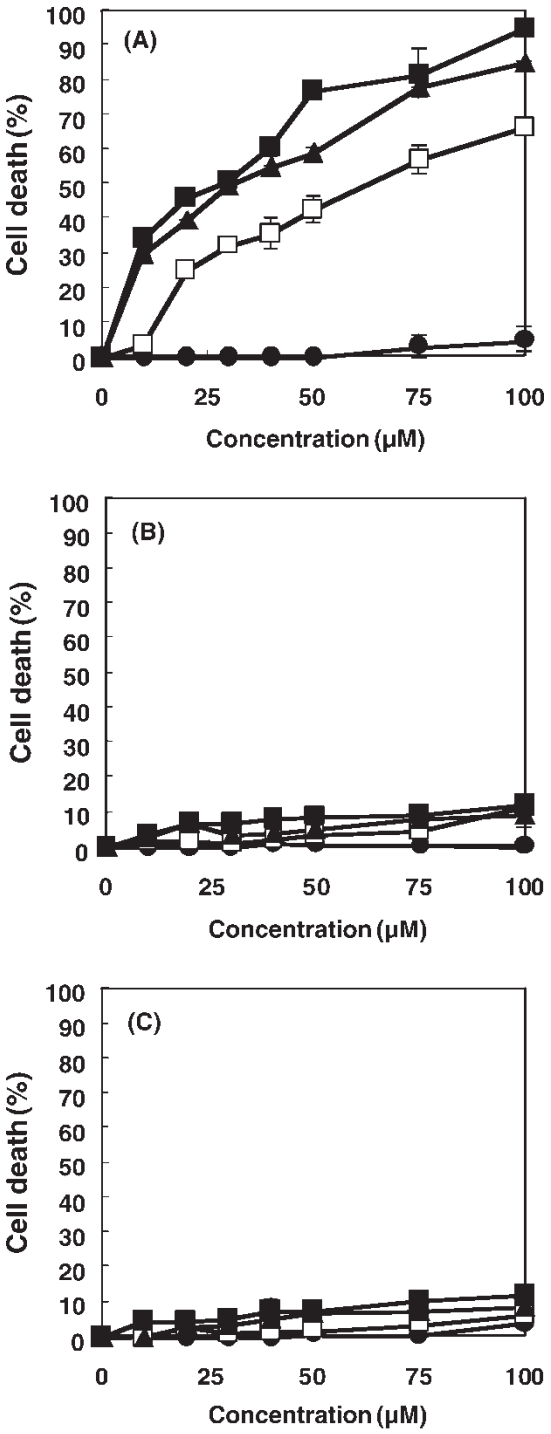

Figure 8. Effects of various fatty acids on the cell death of HeLa (A), L929 (B) and RAW264.7 (C) cells. The cells were exposed to various fatty acids, including LA (closed circles), AA (open squares), EPA (closed triangles) and DHA (closed squares). The cells were exposed to the indicated dose of each fatty acid for 18 h. The cell viability was then estimated from the release of LDH. Data are the means ± SD of three replicates in each case.

with AA, EPA and DHA, but not with LA. In contrast, L929 and RAW264.7 cells were apparently resistant to the cellular death induced by EPA and DHA.

We determined the level of hydroperoxide in mitochondria in order to elucidate the relationship between cellular death and hydroperoxide generation in mitochondria (Figure 9). The level of hydroperoxide in mitochondria was significantly increased in HeLa cells at 4 h after treatment with EPA. On other hand, no enhancement of the hydroperoxide

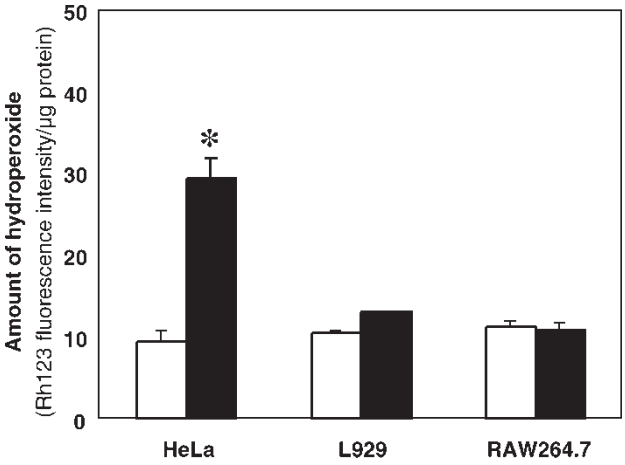

Figure 9. Mitochondrial levels of hydroperoxide in HeLa, L929 and RAW264.7 cells treated with EPA. Mitochondria were isolated from cells treated with (closed bars) or without (open bars) EPA for 4 h. The isolated mitochondria were incubated with DHR and the fluorescence was measured. Data are the means ± SD of three replicates in each case. $*P < 0.05$ was considered significant.

level was observed in mitochondria in L929 and RAW264.7 cells, which were both insensitive to the cellular death induced by EPA and DHA.

Protection of EPA-induced apoptosis by overexpression of PHGPx in mitochondria

Our finding that the mitochondrial hydroperoxide level was elevated in cells induced to undergo apoptosis suggested that the generated hydroperoxide may participate in the initiation of apoptosis. Therefore, we studied the effect of overexpression of PHGPx, an antioxidant enzyme, on the EPA-induced apoptosis by using mitochondrial PHGPx-overexpressing RBL2H3 cells (Figure 10A). Although non-transfected RBL2H3 cells were susceptible to treatment with EPA or DHA, as shown in Figure 1, the apoptosis induced by EPA and DHA was potently suppressed by the mitochondrial PHGPx overexpression. Mitochondrial PHGPx-overexpressing cells resisted cell death after treatment with 100 μM EPA or DHA. Furthermore, the overexpression of mitochondrial PHGPx blocked cytochrome *c* release, AIF release and DNA cleavage after incubation with EPA (data not shown). The difference in EPA effect on the viability of non-transfected RBL2H3 cells and mitochondrial PHGPx-overexpressing RBL2H3 cells was not due to a difference in the rate of EPA uptake into the cells, since no significant difference was observed between the rates of uptake of radioactive EPA into the two cell lines (data not shown).

The generation of hydroperoxide in mitochondria after EPA treatment was effectively suppressed by the overexpression of mitochondrial PHGPx (Figure 10B). This result suggests that the mitochondrial PHGPx may protect against the apoptosis induced by EPA by

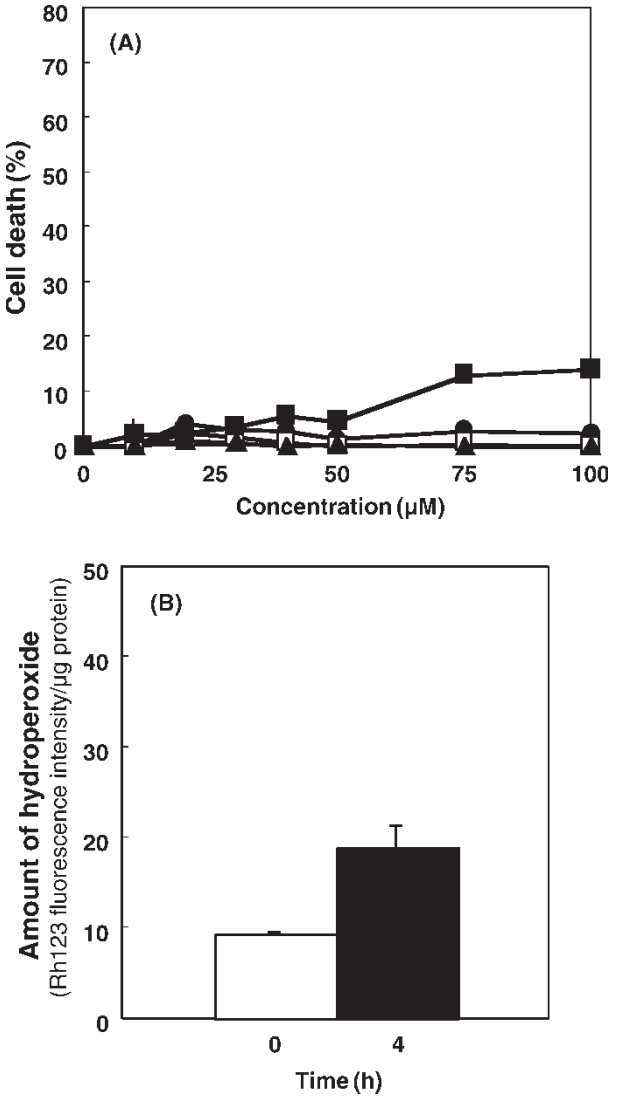

Figure 10. Effect of overexpression of mitochondrial PHGPx on the apoptosis of RBL2H3 cells induced by fatty acids and the EPA-induced hydroperoxide generation in mitochondria. (A) Mitochondrial PHGPx-overexpressing RBL2H3 cells were exposed to the indicated doses of LA (closed circles), AA (open squares), EPA (closed triangles) and DHA (closed squares) for 18 h. The cell viability was then estimated from the release of LDH. (B) Mitochondria were isolated from cells treated with (closed bars) or without (open bars) EPA for 4 h. The isolated mitochondria were incubated with DHR and the fluorescence was measured. Data are the means ± SD of three replicates in each case.

scavenging the hydroperoxide formed in mitochondria in response to EPA stimulation.

Structural requirement of fatty acids for the cytotoxicity

We determined the effects of various fatty acids with different numbers of double bonds and chain lengths on the cellular death (Figure 11). In the 18 carbon chain series, oleic acid (18:1) and linoleic acid (18:2) had no effect on the cell viability, while octadecatetraenoic acid (18:4) induced cell death at 50 μM. In PUFAs with a 20 carbon chain (C20), eicosadienoic

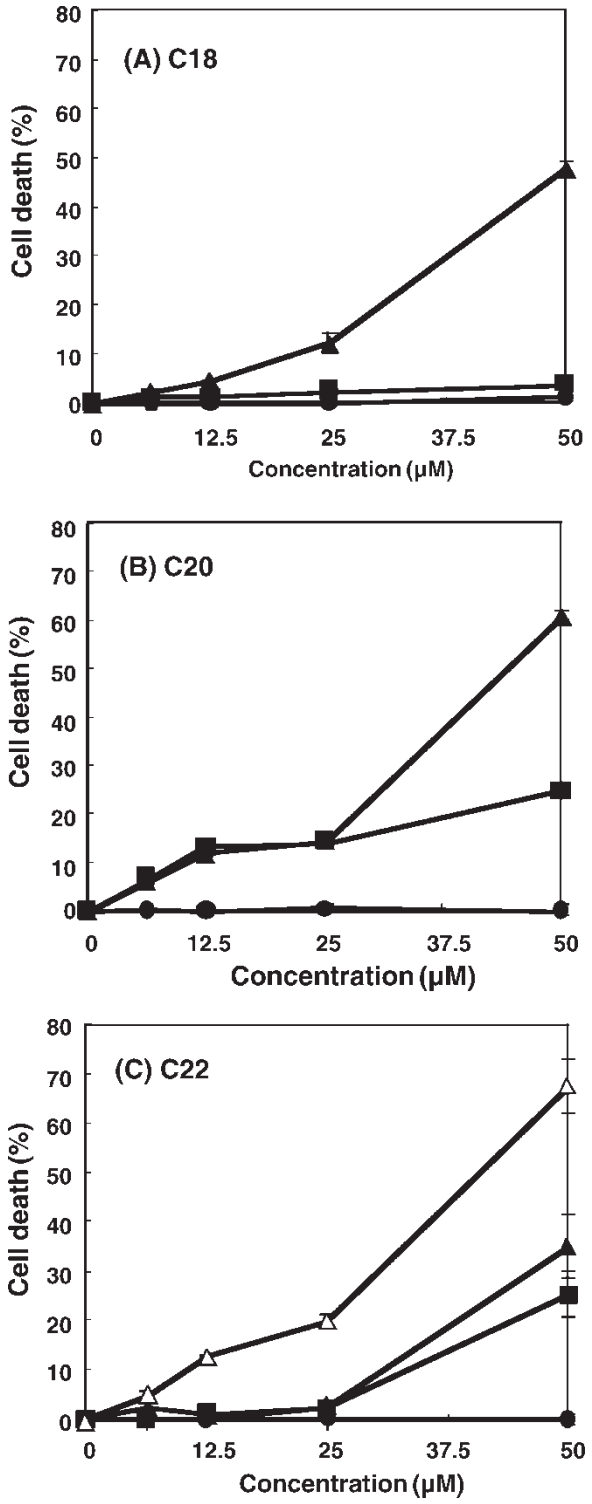

Figure 11. Effects of fatty acids on the cell death of RBL2H3 cells. RBL2H3 cells were exposed to the indicated doses of various fatty acids with different carbon chain lengths and numbers of double bonds for 18 h. (A) C18 fatty acid series, including oleic acid (closed circles), LA (closed squares) and octadecatetraenoic acid (closed triangles). (B) C20 fatty acid series, including eicosadienoic acid (closed circles), AA (closed squares) and EPA (closed triangles). (C) C22 fatty acid series, including docosadienoic acid (closed circles), docosatetraenoic acid (closed squares), docosapentaenoic acid (closed triangles) and DHA (open triangles). The cell viability was estimated from the release of LDH. Data are the means ± SD of three replicates in each case.

acid (20:2) did not induce cell death, while higher unsaturated C20 fatty acids, such as 20:4 and 20:5, induced cell death. In C22 fatty acids, docosadienoic acid (22:2) with 2 double bonds failed to induce cellular death. These results indicate that dienoic acid does not have the ability to induce cellular death, while PUFAs with more than 4 double bonds are cytotoxic to RBL2H3 cells. The production of hydroperoxide was observed in cells by the treatment with PUFAs with more than 4 double bond, while hydroperoxide did not generated by the treatment with dienoic acids (data not shown).

Discussion

Several studies have demonstrated that supplementation of cultured cells with PUFAs enhances the production of ROS [18,31–33]. Human fibroblast enrichment with PUFAs initiated a rise in the intracellular ROS level, as determined by dichlorofluorescein-diactetate (DCFH-DA) and thiobarbituric-reactive substances (TBARS) [33]. After exposure to 60 μM EPA, significant ROS generation was observed in HL-60 cells, as assessed by DCFH-DA measurements [18]. EPA also induced rapid generation of intracellular superoxide and hydroperoxide within 2 h in RBL2H3 cells (Figure 7A,B). Accumulating evidence suggests that ROS may act as mediators of apoptosis [34,35]. For example, hydrogen peroxide can induce apoptosis in a variety of cell types [36], while NO-generating agents cause apoptosis in macrophages [37]. ROS are formed during the cell death triggered by ceramide in B cells [38] and may be mediators of such apoptosis. The intracellular sources of ROS include mitochondrial oxidation, the microsomal cytochrome P450 system and plasma membrane NADPH oxidase. ROS from mitochondria may be responsible for the close association between the activities of mitochondria and cell death [39]. This possibility is supported by several observations. Apoptosis is enhanced by antimycin, which stimulates the production of ROS via inhibition of complex III [40], while TNFα-, Fe^{2+}-, amyloid β-peptide- and alkaline-mediated apoptosis is blocked by manganese superoxide dismutase (Mn-SOD), which scavenges superoxide that has leaked from the mitochondrial respiratory chain [41,42]. Hypoglycemia-induced apoptosis was potently suppressed by overexpression of mitochondrial PHGPx through scavenging of the hydroperoxide in mitochondria [43]. The present study demonstrated that hydroperoxide production was significantly enhanced in mitochondria isolated from RBL2H3 cells after EPA treatment (Figure 7C), and the enhancement was efficiently suppressed by overexpression of mitochondrial PHGPx (Figure 10B). The generation of hydroperoxide in mitochondria after EPA treatment was also found in

HeLa cells induced to undergo apoptosis by EPA, whereas it was not observed in the apoptosis-insensitive L929 and RAW264.7 cells (Figures 8 and 9). The reason why hydroperoxide was not generated in mitochondria of L929 cells and RAW264.7 was not known. However, one of possible explanation due to the different activities of mitochondrial anti-oxidant enzymes.

The mechanism by which EPA induce the production of ROS in mitochondria is not well understood. However, the increase in ROS production might be linked with impaired mitochondrial respiratory activity. Colquhoun and Schumacher found that superoxide anion radical was generated after the induction of apoptosis of Walker 256 rat carcinosarcoma cells by the treatment with EPA and that the treatment of cell with EPA induced the significant decrease in the activities of complexes I + III and IV [44]. Antimycin which is specific complex III mitochondrial inhibitor potentiated the production of ROS in U937 cells treated with ceramide [40]. These results suggest that defect in the mitochondrial respiratory activity would be accompanied the production of ROS in mitochondria.

The crucial role of the hydroperoxide generated in mitochondria in the initiation of apoptosis is further supported by the following observations. First, EPA induced hydroperoxide generation in mitochondria and initiated apoptosis, whereas LA did not stimulate such hydroperoxide formation and failed to induce apoptosis. Second, EPA induced apoptosis and hydroperoxide production in mitochondria in HeLa cells as well as in RBL2H3 cells, whereas such hydroperoxide production was not observed in the apoptosis-resistant RAW264.7 and L929 cell lines. Third, more direct evidence was obtained by using the mitochondrial PHGPx-overexpressing cells. EPA-induced apoptosis was blocked when the accumulation of hydroperoxide in mitochondria was prevented by overexpression of mitochondrial PHGPx. Taken together, these results clearly suggest that the production of hydroperoxide in mitochondria may be an important early trigger of the apoptosis induced by EPA.

Mitochondria play a crucial role in apoptosis through the release of apoptotic factors, such as cytochrome *c*, AIF and Smac/DIABLO, from the intermembrane space into the cytosol [45]. EPA induced the release of cytochrome *c* and AIF from mitochondria in which the hydroperoxide level was enhanced. The mechanism of the liberation of these proapoptotic proteins through the permeability transition (PT) pores of mitochondria remains unclear. The central role of adenine nucleotide translocator (ANT), one of the components and a potential regulator of PT pores, was demonstrated using pharmacological inhibitors of ANT, namely atractyloside and bongkrekic acid [46]. Another previous study reported that peroxidation of cardiolipin, a mitochondria-specific phospholipid, caused inactivation of ANT and induced the opening of PT pores [47]. This possibility was supported by the result that the EPA-induced release of cytochrome *c* and AIF was inhibited by overexpression of mitochondrial PHGPx, which preferentially reduces the phospholipid hydroperoxide. Thus, one possible explanation would be that peroxidation of cardiolipin by EPA treatment may induce ANT inactivation and then open the PT pores. Another possible mechanism would involve the direct interaction of ANT with fatty acids. Schonfeld and Bohnensack demonstrated binding of free fatty acids to ANT using a competitive inhibition study [48], since free fatty acids inhibited the binding of radioactive atractyloside to ANT. The interaction could indicate that free fatty acids promote the mitochondrial permeability transition by stabilizing the "cytosolic" conformation of ANT.

Other apoptosis-related proteins, that are members of the Bcl-2-family of proteins, include both antagonists (Bcl-2 and Bcl-xL) and agonists (Bax and Bad) of apoptosis. Although we evaluated the expression of these Bcl-2-related proteins by immunoblotting in order to characterize the participation of each protein in EPA-induced apoptosis, there were no significant differences in the respective levels of Bcl-2, Bcl-xL, Bax and Bad between non-treated and EPA-treated cells (data not shown).

In the majority of apoptosis, various forms of stress due to apoptotic agents, such as etoposide, staurosporin and UV irradiation, induce the release of cytochrome *c* from mitochondria, which then activates caspase-3 through the formation of apoptotic protease activating factor (Apaf) complexes in the cytosol and induces apoptosis. Heimili et al. observed that EPA caused increased activities of caspase-3 and -9 in Ramos cells, and that inhibitors of caspase-3 and -9 reduced the EPA-induced apoptosis [21]. EPA also activated various types of caspase-like proteases, such as caspase-3,-6, -8 and -9 in HL-60 cells [18]. EPA-induced DNA fragmentation in HL-60 cells was suppressed by treatment with the broad-specificity caspase inhibitor z-VAD-fmk. In the current study, EPA did not significantly enhance caspase-3 activity at 6 h, and z-VAD-fmk did not protect against EPA-induced apoptosis in RBL2H3 cells (data not shown). The reason for this discrepancy is not known, although it may be due to the different characters of the cell types. Furthermore, the apoptosis preceded the caspase-3 activation which had not occurred at 6 h after the addition of EPA. These results therefore indicate that the EPA-induced apoptosis of RBL2H3 cells is independent of the caspase-mediated death pathway.

PARP-1 is a nuclear enzyme that responds to DNA damage and facilitates DNA repair [49]. PARP-1 activation mediates cell death in ischemia-reperfusion injury [50], myocardial infarction [51], glutamate excitotoxicity [52] and ROS-induced injury [53].

Although the molecular mechanism of PARP-1-induced cell death is not fully understood, Yu et al. recently reported that PARP-1 activation is required for AIF translocation from mitochondria to the nucleus, and that AIF is necessary for PARP-1-dependent cell death [30]. They also demonstrated that the specific PARP-1 inhibitor DHIQ blocked *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine-induced toxicity and inhibited AIF translocation, thereby inducing caspase-independent apoptosis. AIF is a caspase-independent death effector released from mitochondria and induces chromatin condensation and large-scale DNA fragmentation [53]. AIF participates in the apoptosis induced by staurosporin, cisplatin, etoposide, ceramide, ATP depletion, dexamethasone, granulysin, cathepsin D and GD3 ganglioside [54–56]. Relocation of AIF from mitochondria to the nucleus was observed in RBL2H3 cells at 4 h after EPA treatment (Figure 4). To evaluate the involvement of PARP-1 and AIF in the EPA-induced apoptosis, we studied the effect of DHIQ on EPA-induced apoptosis which involves AIF translocation from mitochondria to the nucleus. DHIQ prevented the AIF translocation (Figure 6) and inhibited EPA-induced apoptosis to 50% of that in the absence of DHIQ (Figure 5). Incomplete inhibition of apoptosis by DHIQ suggests that the cell death caused by EPA might in part due to the necrosis. These results suggest that EPA partially induces apoptosis in a PARP-1-dependent manner mediated by AIF translocation in RBL2H3 cells.

Further characterization of the initial mechanism involved in EPA-induced apoptosis is important, since PUFAs may play a role in the prevention and treatment of certain types of cancer. All the observations in the study support the hypothesis that hydroperoxide generation in mitochondria may be an important step during EPA-induced apoptosis. Although the detailed mechanism of EPA- induced apoptosis has not yet been fully clarified, the present study provides the first evidence that hydroperoxide production in mitochondria may be an important event.

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