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# Role of calcium-induced mitochondrial hydroperoxide in induction of apoptosis of RBL2H3 cells with eicosapentaenoic acid treatment

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**Abstract**
Eicosapentaenoic acid (EPA) was previously shown to induce caspase-independent apoptosis in rat basophilic leukemia cells (RBL2H3 cells) by translocation of apoptosis-inducing factor (AIF) [Free Radic Res (2005) **39**, 225–235]. Here, we attempted to investigate the mechanism of EPA-induced apoptosis. A rapid and sustained increase in calcium was observed in mitochondria at 2 h after the addition of EPA prior to apoptosis. Coincidently, hydroperoxide was generated in the mitochondria after exposure to EPA. Production of mitochondrial hydroperoxide was significantly reduced by ruthenium red, an inhibitor of mitochondrial calcium uniporter, and BAPTA-AM, a cytoplasmic calcium chelator, indicating that generation of hydroperoxide is triggered by an accumulation of calcium in the mitochondria. The production of mitochondrial hydroperoxide was markedly attenuated by overexpression of phospholipid hydroperoxide glutathione peroxidase (PHGPx) in the mitochondria. Apoptosis was therefore, significantly prevented through inhibition of mitochondrial hydroperoxide generation with mitochondrial PHGPx, ruthenium red or BAPTA-AM. However, accumulation of calcium in the mitochondria was not prevented by mitochondrial PHGPx although apoptosis was blocked, indicating that elevated calcium does not directly induce apoptosis. Taken together, our results show that calcium-dependent hydroperoxide accumulation in the mitochondria is critical in EPA-induced apoptosis.

**Keywords:** *Eicosapentaenoic acid, apoptosis, mitochondria, phospholipid hydroperoxide glutathione peroxidase, hydroperoxide, calcium*

**Abbreviations:** *PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; AIF, apoptosis-inducing factor; PHGPx, phospholipid hydroperoxide glutathione peroxidase; Mn-SOD, Mn-superoxide dismutase; BAPTA-AM, O,O′-Bis(2-aminophenyl)ethyleneglycol-N,N,N′,N′-tetraacetic acid, tetraacetoxy methyl ester; ROS, reactive oxygen species; DHR, dihydrorhodamine 123; MPT, mitochondria permeability transition; LDH, lactate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline*

## Introduction

Dietary polyunsaturated fatty acids (PUFAs) have been suggested as a potential group of natural products that play significant roles in modulating cancer development [1–3]. In animal models, dietary supplementation with fish oil, which is rich in n-3 and n-6 PUFAs, inhibits chemically-induced carcinogenesis [4,5]. The effects of PUFAs on cell growth have also been investigated using a variety of cell lines [6–8], and recent studies have indicated that they play an important role in the regulatory mechanism controlling cellular proliferation and apoptosis [9,10]. The ability of eicosapentaenoic acid (EPA) to induce apoptosis has been demonstrated in several cell lines [11–14]. However, the exact

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mechanism of EPA-induced apoptosis remains unknown.

Disruption of cellular calcium homeostatis has been proposed as a critical event in apoptosis [15]. Increases in cytoplasmic free calcium levels are known to precede cell death in cells undergoing apoptosis [16,17] and are sufficient to induce apoptosis in a number of different cell types [18,19]. Agents that suppress this calcium influx can therefore prevent apoptosis in several different systems [17,20]. Mitochondria play important roles in regulating cellular calcium homeostasis [21,22] and previous findings revealed associations between mitochondrial reactive oxygen species (ROS) production and disruption of calcium homeostasis in cells undergoing apoptosis [21].

Mitochondria are a major physiological source of ROS, which are generated during mitochondrial respiration, and ROS generated in excess might therefore act as mediators of the apoptotic signaling pathway. The production of ROS induced by tumor necrosis factor α (TNF-α), ceramide, staurosporine and hypoglycemia has been proposed as an early event in the induction of apoptosis [17,23–25]. Mitochondrial ROS production is regulated by a number of antioxidant enzymes including phospholipid hydroperoxide glutathione peroxidase (PHGPx), classical glutathione peroxidase (cGPx) and Mn-superoxide dismutase (Mn-SOD), and overexpression of these antioxidant enzymes in the mitochondria suppresses apoptosis. Overexpression of Mn-SOD was shown to completely suppress TNF-α induced and amyloid-β-peptide-induced apoptosis in MCF-7 and PC6 cells, respectively [26,27]. Apoptosis induced by hypoglycemia, etoposide and staurosporine was also potentially inhibited by overexpression of mitochondrial PHGPx [24].

In a previous study, we found that EPA induced apoptosis through a caspase-independent pathway while EPA-induced apoptosis could be involved with hydroperoxide in the mitochondria [28]. This evidence suggests that mitochondria derived ROS might be involved in the induction of apoptotic death. However, details of the mechanism responsible for induction of apoptosis remain to be clarified. Here, we demonstrate that EPA induces the uptake of calcium into the mitochondria and hydroperoxide generated by calcium in the mitochondria triggers the initiation of apoptosis.

## Materials and methods

### *Reagents*

Dihydrorhodamine 123 (DHR) was obtained from Molecular Probes Inc. (Leiden, The Netherlands), RNase was purchased from Roche Molecular Biochemicals (Almere, The Netherlands) and BAPTA-AM was obtained from Dojindo Laboratories. (Kumamoto, Japan). The anti-cytochrome c antibody was obtained from PharMingen Inc. (San Diego, CA), the anti-AIF antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and HRP-conjugated rabbit anti-mouse IgG was purchased from Zymed Lab. (San Diego, CA). EPA and ruthenium red were obtained from Funakoshi Pharmaceutical Co. Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan) and Block Ace was obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). All other chemicals were obtained from WAKO Pure Chemicals Ind. Ltd. (Tokyo, Japan).

### *Cell culture*

RBL2H3 cells were maintained in DMEM containing 5% fetal calf serum (FCS). Cells were grown in a humidified incubator at 37°C and used for assays during the exponential phase of growth. We also used a previously established strain of RBL2H3 cells in which PHGPx was overexpressed in the mitochondria [29].

### *Assessment of cell viability*

RBL2H3 cells and mitochondrial PHGPx-overexpressing RBL2H3 cells were plated at $1 \times 10^4$ 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) and pretreated with 60 μM of ruthenium red or 50 μM of BAPTA-AM for 3 h. Non-treated cells, ruthenium red or BAPTA-AM-treated cells and mitochondrial PHGPx-overexpressing cells were suspended in 100 μl of DMEM containing EPA dissolved in ethanol and incubated for an appropriate period in a humidified incubator at 37°C. Cell viability was then determined by measuring the release of lactate dehydrogenase (LDH) as described previously [28].

### *Measurement of calcium in cells and mitochondria*

Fluo 3-AM is often used for calcium indicater [30]. RBL2H3 cells ($5 \times 10^5$ cells/well) were plated in a 12-well plate and then 5 μM of Fluo 3-AM was incorporated into the cells by incubation for 30 min at 37°C under dark conditions in order to determine the intracellular calcium. After the incorporation of Fluo3-AM, cells were washed with PBS. Cells loaded with Fluo3 were then treated with 50 μM EPA for appropriate periods and the fluorescence of Fluo 3 was determined using a fluorometer (Ex: 485 nm, Em: 530 nm).

Mitochondria were isolated from $7 \times 10^7$ cells after exposure to 50 μM EPA for 3 h to determine the level of calcium in the mitochondria [29]. Purified

mitochondria were incubated with 5 μM Fluo 3-AM in 600 μl of PBS for 30 min at 37°C. After incubation, 50 μl aliquots from the mitochondrial suspension were used to determine the level of mitochondrial calcium by the measurement of the fluorescence of Fluo 3.

*Measurement of hydroperoxide in mitochondria*

To assess the levels of mitochondrial hydroperoxide, we used DHR, which is oxidized by hydrogen peroxide and lipid hydroperoxide yielding fluorescent rhodamine 123 [24]. Cells ($7 \times 10^7$ cells) were incubated for 3 h with 60 μM ruthenium red or 50 μM BAPTA or without. After treatment, cells were washed with PBS and treated with 50 μM EPA for 4 h. Following this, mitochondria were isolated from cells and washed three times with PBS. Aliquots of mitochondria (0.1 mg protein) were incubated with 2 μg/ml DHR for 30 min in PBS then fluorescence was monitored using a CytoFluor plate-reader.

*Release of cytochrome c and apoptosis-inducing factor (AIF)*

Cells ($1 \times 10^7$ cells) were treated with 50 μM EPA for 4 h. After the incubation, cells were precipitated and cell pellets were treated with 1.5 ml digitonin (10 μg/ml) for 5 min. Supernatants of the cell lysates after digitonin treatment were collected by centrifugation (20,000*g* 20 min). Cytochrome c and AIF released from mitochondria into the cytosol were detected as follows; Proteins in the supernatant were precipitated by addition of trichloroacetic acid and precipitated proteins was fractionated by SDSPAGE under non-reducing conditions. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Belford, MA), which was then incubated with anti-cytochrome c and anti-AIF antibodies. Treated PVDF membranes were subsequently incubated with HRP-conjugated anti-mouse IgG antibodies, and antibodies binding to the PVDF membrane were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

*Protein quantification*

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

## Results

Levels of cellular and mitochondrial calcium were measured using the fluorescent probe Fluo 3-AM (Figure 1). Fluorescence increased as a function of time, reflecting elevated intracellular levels of calcium in RBL2H3 cells with EPA treatment.

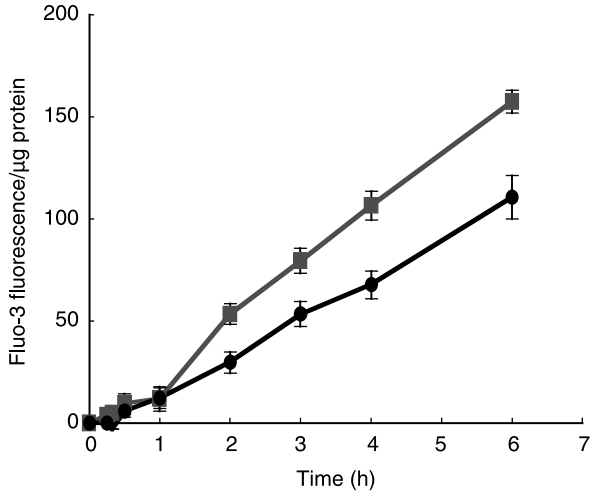


Figure 1. Effect of EPA on calcium uptake into RBL2H3 cells and mitochondria. RBL2H3 cells loaded with Fluo3-AM were incubated with 50 μM of EPA for the indicated times then the fluorescence was determined (closed squares). Mitochondria were isolated from RBL2H3 cells after exposure to 50 μM EPA then incubated with Fluo 3-AM for determination of fluorescence (closed circles). Fluorescence intensity in EPA-treated cells is expressed relative to the intensity in untreated cells. Values represent the average of triplicate results.

At 2 h, an increase in calcium levels in whole cells was detected, and calcium linearly increased up to 6 h after exposure to EPA. A sustained rise in calcium in whole cells secondarily enhanced the accumulation in mitochondria. Calcium in the mitochondria remained low during the first 2 h of exposure to EPA. However, after this, a large increase was observed and this increase continued up to 6 h after the addition of EPA.

Accumulation of mitochondrial ROS is thought to play a role in many cases of cell death. We therefore, employed the fluorescent probe DHR to detect hydroperoxide in mitochondria isolated from EPA-treated cells. Levels of mitochondrial hydroperoxide increased following exposure of RBL2H3 cells to EPA and the level after 4-h EPA treatment was 4.5 times higher than that of non-treated mitochondria (Figure 2). Production of hydroperoxide in mitochondria of EPA-treated RBL2H3 cells was markedly suppressed by overexpression of mitochondrial PHGPx.

To examine whether formation of mitochondrial hydroperoxide is calcium-dependent, we assessed the production of mitochondrial hydroperoxide after EPA exposure using ruthenium red, an inhibitor of calcium uniporter, or BAPTA-AM, a chelater of calcium (Figure 2). The generation of mitochondrial hydroperoxide was potentially reduced by inhibited uptake of calcium into the mitochondria with ruthenium red and BAPTA-AM treatment, indicating that the accumulation of calcium in mitochondria induces the production of hydroperoxide.

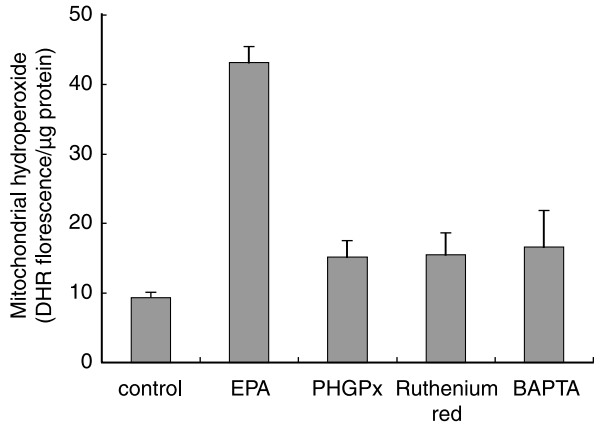


Figure 2. Effects of overexpression of mitochondrial PHGPx, ruthenium red and BAPTA-AM on production of hydroperoxide in mitochondria of EPA-treated cells. Cells were pretreated for 3 h in the presence or absence of ruthenium red or BAPTA-AM. Control cells, treated cells and mitochondrial PHGPx-overexpressing cells were then exposed to 50 μM EPA for 4 h. Mitochondria were isolated from the cells then levels of mitochondrial hydroperoxide were determined using DHR. Values represent the means ± S.D. of five replicates in each case.

We also assessed the effect of overexpression of mitochondrial PHGPx on the accumulation of calcium in the mitochondria to examine whether generation of hydroperoxide in the mitochondria plays a role (Figure 3). The uptake of calcium caused by EPA treatment was not reduced by overexpression of mitochondrial PHGPx, while calcium uptake into the mitochondria was markedly suppressed by ruthenium red and BAPTA-AM treatment. These results indicate that accumulation of calcium in the mitochondria occurs upstream of mitochondrial hydroperoxide formation.

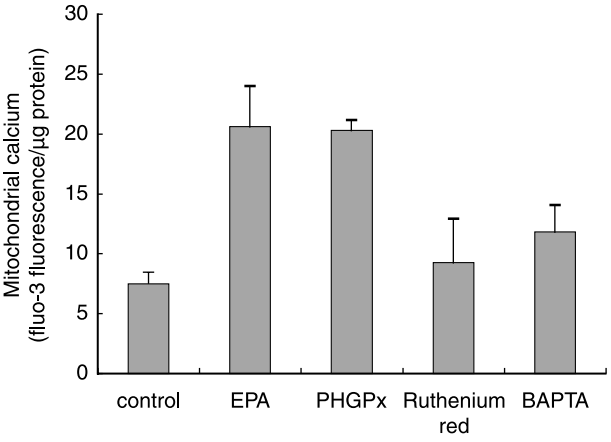


Figure 3. Effects of overexpression of mitochondrial PHGPx, ruthenium red and BAPTA-AM on the EPA-dependent uptake of calcium into mitochondria. Cells were pretreated for 3 h in the presence or absence of ruthenium red or BAPTA-AM. Control cells, treated cells and mitochondrial PHGPx-overexpressing cells were then exposed to EPA for 4 h. Mitochondria were isolated from the cells then levels of mitochondrial calcium were determined using Fluo 3-AM. Values represent the means ± S.D. of five replicates in each case.

Western blot analysis was conducted to examine the effect of mitochondrial PHGPx and ruthenium red on the release of cytochrome c and AIF from mitochondria in EPA-treated RBL2H3 cells. AIF and cytochrome c were both released and detected in the cytoplasmic fraction at 4 h after exposure to EPA. Overexpression of mitochondrial PHGPx and treatment with ruthenium red blocked EPA-induced release of cytochrome c and AIF from the mitochondria (Figure 4).

In previous report, we demonstrated that DNA fragmentation of RBL2H3 cells was induced at 18 h after the treatment with EPA [28]. Apoptosis of RBL2H3 cells were induced by treatment with EPA (Figure 5). Number of dead cells increased gradually after incubation with EPA for 6 h, reaching 60% of the total. The role of mitochondrial hydroperoxide in the induction of apoptosis was examined under suppression of mitochondrial hydroperoxide. As shown in Figure 5, apoptosis was blocked by overexpression of mitochondrial PHGPx and also by the treatment with ruthenium red or BAPTA-AM, indicating that generation of hydroperoxide in the mitochondria plays a crucial role in initiating EPA-induced apoptosis.

## Discussion

EPA was previously shown to induce apoptosis of RBL2H3 cells [27]; however, the key events implicated in EPA-triggered apoptosis remained unknown. In this study, EPA induced generation of hydroperoxide in mitochondria prior to induction of apoptosis. Several studies have demonstrated that supplementation of cultured cells with PUFAs enhances the production of ROS [11,31,32]. Significant ROS generation was also observed in HL60 cells after treatment with EPA [11]. Particularly, ROS from mitochondria might therefore be responsible for the close association between mitochondria activity and

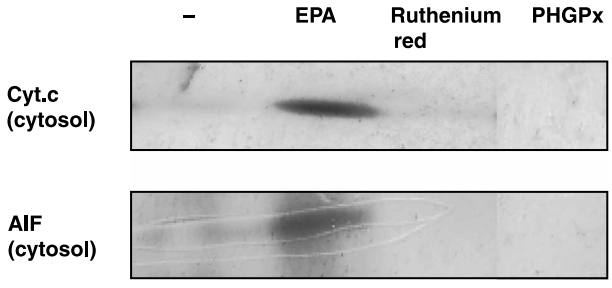


Figure 4. Effect of ruthenium red and overexpression of mitochondrial PHGPx on the EPA-induced release of cytochrome c and AIF from mitochondria. RBL2H3 and mitochondrial PHGPx-overexpressing cells were preincubated in the presence or absence of ruthenium red for 3 h then incubated with 50 μM EPA for 4 h. AIF or cytochrome c in the cytosol was then detected by immunoblotting with an anti-AIF or anti-cytochrome c antibody.

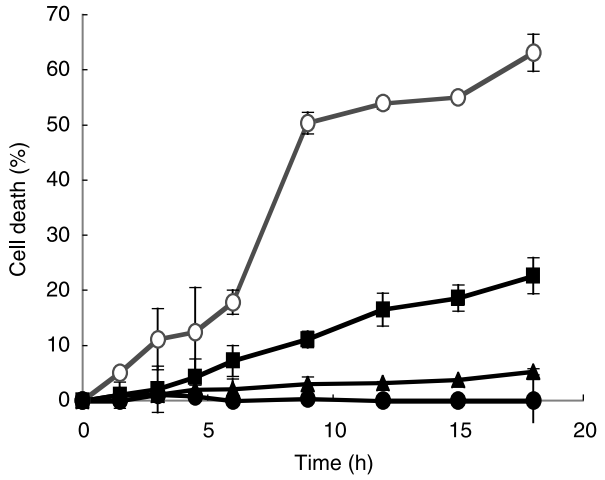


Figure 5. Effects of mitochondrial PHGPx, ruthenium red and BAPTA-AM on the apoptosis of RBL2H3 cells. Cells were pretreated for 3 h in the presence or absence of ruthenium red or BAPTA then exposed to EPA for the indicated times. Cell death of EPA-treated cells (open circles), EPA-treated mitochondrial PHGPx-overexpressing cells (closed circles), EPA and ruthenium red-treated cells (closed triangles), and EPA and BAPTA-AM treated cells (closed squares) was determined by measuring LDH release. The percentage of cell death is expressed relative to that of untreated cells. Values represent the average of triplicate results.

cell death [33]. This possibility is supported by observations that apoptosis was prevented by overexpression of antioxidant enzymes such as mitochondrial PHGPx [24] and Mn-SOD [26,27]. Moreover, mitochondrial production of ROS is thought to contribute significantly to neuronal cell death caused by excitotoxicity and various acute and chronic neurological disorders, such as cerebral ischemia/reperfusion and Parkinson's disease [34,35]. In the present study, we also demonstrated that liberation of AIF from mitochondria and the induction of apoptosis were prevented by reduced generation of mitochondrial hydroperoxide by overexpression of mitochondrial PHGPx or treatment with ruthenium red. The present and previous results indicate that generation of hydroperoxide in mitochondria is a critical event triggering EPA-induced apoptosis.

Present study demonstrates that EPA induced a significant elevation in cellular and mitochondrial calcium at 2 h after treatment. Several reports have also shown that changes in calcium homeostasis are linked to the initiation of apoptosis [15–19]. A recent report also demonstrated that calcium can trigger nuclear translocation of AIF in neuronal cells and human hepatocarcinoma cells [36,37]. These results are consistent with the present results demonstrating that prevention of the calcium influx into mitochondria by ruthenium red inhibits the liberation of AIF from mitochondria with EPA treatment. The accumulation of calcium in mitochondria is also known to induce dissipation of mitochondria permeability transition (MPT) [38]. EPA-induced apoptosis is thought to be related to calcium-induced alternation of mitochondrial homeostasis, since apoptosis is inhibited by treatment with ruthenium red or BAPTA-AM. This result indicates a role for calcium in EPA-induced apoptosis, suggesting a novel mechanism of EPA-induced apoptosis.

Bcl-2 family proteins play a fundamental role in the integration of pro-apoptotic and anti-apoptotic signals, controlling intracellular calcium transport. Recent evidence suggests that overexpression of Bcl-2 results in a reduction in calcium in the endoplasmic reticulum, the most important calcium storage compartment in the body, resulting in enhanced mitochondrial calcium uptake, whereas down-regulation of Bcl-2 yields an increase in calcium in the endoplasmic reticulum [39–41]. Similar to Bcl-2, overexpression of Bax or Bak has been reported to induce depletion of calcium in the endoplasmic reticulum [42]. Using Western blotting, we examined whether the accumulation of calcium is due to changes in the expression of Bcl-2 family proteins; however, expression of Bcl-2 and Bax was not altered with EPA treatment (data not shown). Calcium mobilization by EPA is therefore not associated with changes in the expression of Bcl-2 family proteins.

A rapid and sustained increase in mitochondrial calcium and a coincidental production of hydroperoxide in the mitochondria were observed in EPA-treated cells. The linkage between mitochondrial calcium uptake and ROS generation was demonstrated by the inhibition of EPA-induced mitochondrial hydroperoxide production with ruthenium red or BAPTA-AM treatment, indicating that mitochondrial hydroperoxide formation is dependent on the accumulation of calcium. It is noteworthy that hydroperoxide production and apoptotic induction were almost completely abolished by the overexpression of mitochondrial PHGPx; however, uptake of calcium into the mitochondria was not prevented. These results indicate that elevated mitochondrial calcium does not directly induce apoptosis, but rather, calcium-dependent mitochondrial hydroperoxide formation is the primary event responsible for EPA-induced apoptosis.

Recent findings have shown that calcium-overloaded mitochondria exhibit an increase in ROS production. For example, generation of ROS has been reported in isolated mitochondria by addition of calcium, inorganic phosphate and respiratory substrates [42–45]. However, the exact mechanism by which calcium induces the generation of mitochondrial hydroperoxide is still not clearly understood. One possible explanation is that calcium-induced changes in membrane organization occur as a result of binding with cardiolipin, a negatively charged phospholipid in the inner mitochondrial membrane and tightly bound to various protein complexes including proteins of the electron transport chain such as complex III [46,47]. Grijalba et al. demonstrated lipid packing and the

formation of a lipid domain in submitochondrial particles or cardiolipin-containing liposomes [48]. This result suggests that calcium induces the formation of immobilized clusters of a membrane protein tightly bound to cardiolipin, with rearrangement of this protein as a result of calcium-cardiolipin interactions impairing the flow of electrons within the electron transport chain at a site within complex III, which is proximal to the site of ROS generation [48]. Another possible mechanism of calcium-induced hydroperoxide in mitochondria might involve cytochrome c. Cytochrome c oxidized linoleic acid with lipoxygenase activity in the presence of calcium [49], and lipoxygenase activity of cytochrome c was remarkably low in the absence of calcium. Oxidized linoleic acid was also shown to form in submitochondrial particles in the presence of calcium. This finding suggests that cytochrome c could peroxidize mitochondrial lipid as a result of the calcium influx into the mitochondria with EPA treatment.

In this study, AIF and cytochrome c release were prevented with suppressed generation of mitochondrial hydroperoxide. Opening of MPT pores is known to be a prerequisite for the release of cytochrome c and AIF from mitochondria. The observation that MPT was stimulated by prooxidants suggests that MPT could be caused by ROS produced by the mitochondrial respiratory chain [43]. The molecular mechanism of ROS-induced MPT stimulation is unknown; however, one possibility is that phospholipid hydroperoxide might play a role in opening MPT pores, since release of AIF is inhibited by the overexpression of mitochondrial PHGPx whose primary substrate is phospholipid hydroperoxide. Cardiolipin is rich in polyunsaturated fatty acid compared with other mitochondrial phospholipids, suggesting that ROS are sensitive to cardiolipin. Peroxidation of cardiolipin induces decreased activity of complexes I and IV, which might induce a loss of mitochondrial membrane potential [50]. Our previous study demonstrated that peroxidation of cardiolipin caused inactivation of the ADP/ATP translocator and the opening of MPT pores [51]. Another possibility is that MPT is related to membrane protein thiol status. MPT is prevented by thiol reductants such as dithiothreitol [52], but promoted by oxidants such as diamide [53], suggesting that MPT is caused by protein modification of thiol residues with ROS. These previous results suggest that the generation of hydroperoxide in mitochondria could play a pivotal role in the liberation of proapoptotic factors from mitochondria and the induction of EPA-induced apoptosis.

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