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Docosahexaenoic acid induces apoptosis in CYP2E1-containing HepG2 cells by activating the c-Jun N-terminal protein kinase related mitochondrial damage

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Abstract

Docosahexaenoic acid (DHA) causes apoptosis of various cancer cells, but the mechanism of DHA-induced cell death is still unclear. We hypothesized that the early signaling of apoptosis may be important in causing cell death as well as the production of free radical metabolites. DHA caused time- and dose-dependent cell death in human HepG2 hepatoma cells transduced with CYP2E1 (E47) but not in C34 (without CYP2E1), suggesting an important role of CYP2E1 in the DHA-mediated damage. DHA increased the c-Jun N-terminal protein kinase (JNK) activity until 8 h without activating other mitogen-activated protein kinases. The contents of proapoptotic Bad and FasL at 4 h and cytochrome *c* and caspase 3 activity at 8 h were increased and accompanied by the JNK activation in a successive manner. In contrast, Bax and Bcl-2 were not changed. Levels of lipid peroxides (LPOs) were elevated three- and fivefold at 8 and 24 h, respectively, in DHA-induced E47 cells. However, pretreatment with chlormethiazole (CMZ), a specific inhibitor of CYP2E1, significantly reduced the levels of LPO, CYP2E1, JNK activity and the rate of cell death. In addition, pretreatment with quercetin (one as a JNK inhibitor and one as an antioxidant) significantly reduced the cell death rate and JNK and SEK-1 activities. Our results indicated that DHA-mediated apoptosis in E47 cells was induced through the activation of the JNK-related cell death pathway, which may be involved in the production of LPO or reactive oxygen species during the CYP2E1 catalytic cycle, followed by mitochondrial injury and apoptosis.
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Keywords: DHA; Apoptosis; HepG2 cells; CYP2E1; Lipid peroxides (LPOs); JNK activation; MAP kinases

1. Introduction

Docosahexaenoic acid (DHA), a *w-3* fatty acid, is present in high amounts as a major component of brain gray matter and of the retina in most mammalian species [1] and is considered essential for normal neurological and cellular developments. Dietary DHA has been demonstrated to reduce the incidences of atherosclerosis, cardiac arrhythmia and coronary heart diseases [2]. Furthermore, DHA is known to lower the incidence of cancer when it is used alone or combined with other components of dietary fish oils such as eicosapentaenoic acid (EPA). DHA and EPA have been demonstrated to decrease tumor cell proliferation in both in vitro and in vivo models [3–5].

There are several mechanisms by which DHA might induce apoptosis in cancer cells. DHA can cause translocation of phosphatidylserine, which is an early event in apoptosis followed by protein-phosphatase-mediated processes and the systematic destruction of cellular proteins by caspase 3 in apoptotic events [6]. Alternatively, it is possible that the alteration of mitogen-activated protein (MAP) kinases plays an important role in DHA-mediated apoptosis, as demonstrated by the DHA-mediated apoptosis of vascular smooth muscle cells through stimulation of p38 MAP kinase [7]. The activation of extracellular signal-regulated kinase (ERK) is usually associated with cell growth and survival pathways, whereas c-Jun N-terminal protein kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAP kinase are related to cell death. Because of this generally accepted theory [8,9], we hypothesized that MAP kinases may play a key role in the DHA-induced apoptosis of HepG2 cells, although the role of MAP kinases

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in DHA-mediated damage in hepatoma cells has not been studied.

Ethanol-inducible P450 2E1 (CYP2E1, a loosely bound P450 enzyme), present in the liver and other extrahepatic tissues, is known to metabolize various small molecules of potentially toxic substrates: ethanol, acetaldehyde, dimethyl nitrosamine, acetaminophen, CCl_4, benzene, unsaturated fatty acids and so forth [10]. CYP2E1-mediated metabolism of these substrates usually leads to the production of more toxic or carcinogenic metabolites such as reactive or free radical metabolites and lipid peroxides (LPOs) [10–12]. Cederbaum et al. established the stable human E47 HepG2 hepatoma cell line (transduced CYP2E1) to study the role of CYP2E1 in cell damage caused by its substrates, including fatty acids [13–16]. Arachidonic acid (AA; *w-6* fatty acid), as CYP2E1 substrates, was shown to induce much more damage to E47 cells than C34 control hepatoma cells, demonstrating the role of CYP2E1 in the metabolic activation and the subsequent cell damage caused by CYP2E1 substrates [15].

Despite extensive studies on apoptosis of cancer cells caused by DHA [3,17–21], the early signaling mechanism important in DHA-mediated cell death pathway has not been studied systematically. The goal of this study was to investigate the cytotoxic effects of DHA on HepG2 cells expressing CYP2E1 (E47 cells) and the roles of CYP2E1 in the apoptotic mechanisms of the DHA-mediated E47 cells, compared with C34 control cells. In addition, the importance of the levels of LPO in the DHA-mediated apoptosis is compared with that of the early signaling pathway to establish the linkages between CYP2E1, DHA, LPO and cytotoxicity.

2. Materials and methods

2.1. Materials

Minimal essential media (MEM) and other materials for cell culture including gentamicin, antibiotics, L-glutamine and fetal bovine serum (FBS) were purchased from Invitrogen (San Diego, CA, USA). C34 and E47 HepG2 cells were kindly provided by Dr. A. Cederbaum (Mount Sinai Medical School, New York, NY). MTT cell proliferation kit was purchased from Roche Diagnostics GmbH (Indianapolis, USA). LPO kit and SP100625 were purchased from Calbiochem Co. (San Diego, USA). DHA, quercetin, indomethacin, eicosatetraenoic acid (ETYA) and BAPTA-AM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for cytochrome *c*, Bad, Bax and Bcl-2 or conjugated second antibodies were purchased from Pharmingen (San Diego, CA, USA). Antibodies for FasL, JNK, ERK, ATF-2, SEK-1, p38 kinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The caspase 3/7 assay kit was obtained from Promega (Madison, WI, USA). Chlormethiazole (CMZ) was provided by Dr. Joong-Ick Yang (Dong-A Pharmaceutical Company, Seoul, Korea). The other materials not specified were the same as previously described [22].

2.2. Cell culture and fatty acid supplements

Both C34 and E47 cells were maintained in MEM with glutamine, 10% FBS and antibiotics (gentamicin sulfate) at 37°C in a humidified incubator containing 5% CO_2. To study the effects of DHA on cell growth rate, we exposed E47 and C34 hepatoma cells to different concentrations of DHA for 12, 24, 36 or 48 h. DHA completely dissolved in ethanol was dried under argon gas and immediately exposed to 100% FBS as previously described [23]. Final concentrations of fatty acids and FBS in media were 25 to 100 μmol/L and 1%, respectively.

2.3. Cell cytotoxicity

Viability of E47 and C34 cells after the treatment with fatty acids was determined by following the protocol supplied in the CellTiter 96 NonRadioactive Cell Viability Assay Kit (Promega) by using MTT as a substrate.

2.4. TUNEL assay

E47 cells (1×10^4/well) were grown in 8-well chamber glass slides with 50 μmol/L DHA for 12 and 24 h. After the cells were incubated, each slide was detached from the media chamber and immersed in 4% methanol-free formaldehyde in PBS for 25 min at 4°C. Cells were permeated in 0.2% Triton X-100 with protease K and incubated with equilibration buffer including nucleotide mix and TdT enzyme at 37°C for 1 h. Propidium iodide was used for nuclear staining of the cells in mounting medium. Localized green fluorescence of apoptotic cells (Fluorescein-12-dUTP) against red or blue backgrounds (propidium iodide) was visualized by fluorescence microscopy at 520±20 nm after washing the cells with deionized water.

2.5. Measurement of LPOs

Both 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) end products derived from peroxidation of PUFA were measured as convenient indices for lipid peroxidation after culturing the cells under the same conditions. Whole cell lysates from 3×10^6 cells were prepared via three repeated cycles of freeze/thaw in distilled water. Diluted samples (200 μl volume) were added into 650 μl of 10.3 mmol/L *N*-methyl 2-phenylindole. Methane sulfonic acid (15.4 mol/L; final volume, 150 μl) was added to mix the sample, which was then incubated at 45°C for 40 min. After cooling on ice, 4-HNE and MDA products were measured at 596 nm in comparison to the standard curves.

2.6. Western blotting

Whole cell extracts from DHA-treated or untreated cells (3×10^6 cells) were prepared in a buffer, which contains 10 mmol/L Tris–HCl, 2 mmol/L EDTA, 1 μmol/L leupeptin, β-mercaptoethanol, 2 mmol/L EDTA, α-2-macroglobulin, 100 μmol/L PMSF and 250 mmol/L sucrose. After the

extracts were centrifuged (14,000×g for 20 min), the resulting supernatants were subjected to immunoblot analyses against each target protein such as FasL, Bax, Bad, Bcl-2, cytochrome c or CYP2E1. GAPDH was used as a control protein. Each immunoreactive antigen was visualized by using the SuperSignal West Pico ECL kit (Pierce Chemicals) and exposed to X-ray film (Kodak XAR) to identify the target proteins.

2.7. Measurement of MAP kinase activities

E47 cells were treated with 50 μmol/L DHA as indicated, harvested and homogenized in ice-cold lysis buffer as previously described [22]. Cell debris and participate fraction were removed by centrifugation at 14,000×g for 10 min at 4°C. The activity of JNK1 and other MAP kinases in the soluble fraction (300 μg per reaction) was measured using the published method [24].

2.8. Determination of caspase 3/7 activities

Using the Apo-One Homogeneous caspase 3/7 assay kit from Promega, the amount of fluorescent product generated is proportional to those of caspase 3/7 cleavage producers in the sample. E47 cells were grown at a density of 2×10^5 cells/ml in each 96-well plate. After DHA treatment for the indicated times, the caspase 3/7 reagent was added into each well of the plate at a 1:1 reagent-to-sample ratio. After incubation for 5 h at 37°C, the fluorescence of each well was determined at excitation and emission wavelengths of 485±20 and 530±25 nm, respectively.

2.9. Statistical analysis

Experimental results shown were repeated two or three times, unless otherwise indicated. Statistical analyses were performed using Student's t test for the comparisons between two groups and one-way ANOVA test for the comparisons among three or more groups. $P<.05$ was considered statistically significant.

3. Results

3.1. DHA concentration- and time-dependent apoptosis

To study the effect of DHA on growth rates of hepatoma cells with (E47) or without CYP2E1 (C34), we determined the dose- and time-dependent changes in cell morphology and cell viability. Generally, more cells died after longer exposure and higher concentrations of DHA than shorter exposure and lower DHA concentrations. For instance, less than 5% of E47 cells died after exposure to 25 μmol/L DHA for 12 h, 25% died after exposure to 100 μmol/L DHA for 12 h or 50 μmol/L DHA for 24 h and more than 65% of the cells died when they were exposed to 100 μmol/L DHA for 48 h. However, necrosis seemed to take place at higher concentrations of DHA, suggesting that DHA causes both apoptosis and necrosis of E47 HepG2 hepatoma cells, depending on DHA concentration and exposure time. In contrast, less than 15% of C34 cells died after exposure to 50 μmol/L DHA for 12, 24 and 48 h (Fig. 1A). These results suggest that CYP2E1 in E47 cells is involved in the DHA-mediated damage to E47 cells. Because of the considerable damage from apoptosis observed with 50 μmol/L DHA, we used this concentration of DHA in our subsequent experiments.

E47 cells underwent apoptosis with characteristic nuclear shrinkage after exposure to 50 μmol/L DHA for 12 and 24 h. DHA-mediated apoptosis of E47 cells was also confirmed by the appearance of typical DNA fragmentation and nuclear staining, both of which are hallmarks of apoptosis. Maximal DNA fragmentation took place after exposure to 50 μmol/L DHA for 24 h (data not shown). Consistent with these results, E47 cells treated with 50 μmol/L DHA for 12 and 24 h

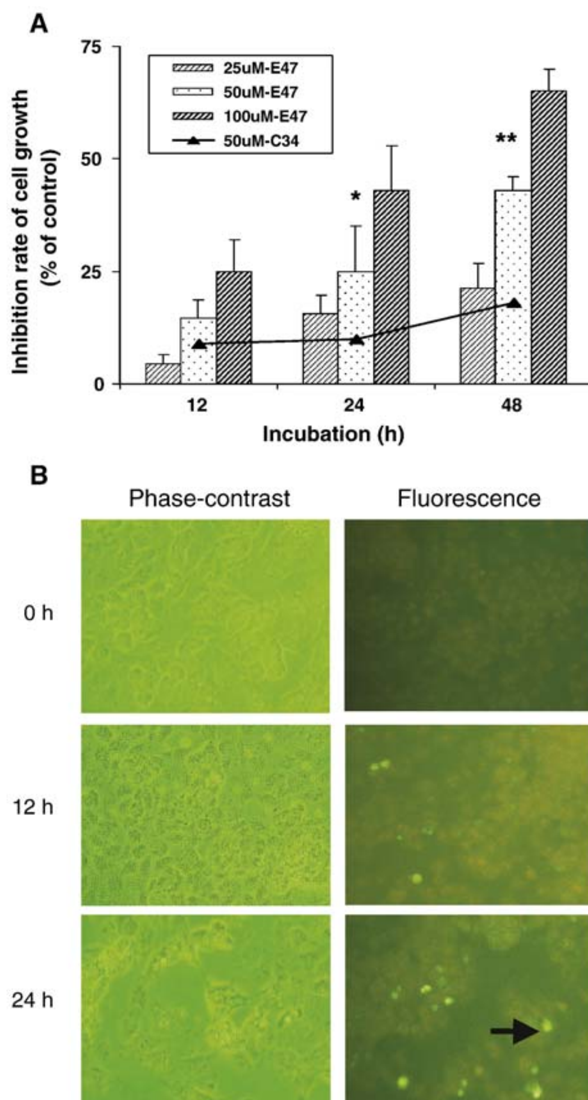

Fig. 1. Time- and dose-dependent effects of DHA on the retardation rate of cell growth in E47 cells compared with C34 cells. (A) MTT reduction assay was significantly different between 50 μmol/L DHA with CYP2E1 (E47) and that without CYP2E1 (C34). More E47 cells died after longer exposure and higher concentration of DHA (*$P<.05$, **$P<.01$). (B) TUNEL assay upon exposure to DHA for 0, 12 and 24 h. The arrow in the right column indicates apoptotic cells compared with cell morphology.

displayed highly fluorescent condensed nuclei (TUNEL assay) in comparison to untreated E47 cells (Fig. 1B).

3.2. Apoptosis-related proteins and activation of caspase 3/7

Changes in the expression of various proapoptotic and antiapoptotic proteins are also important in the progression of apoptosis [25]. We determined the levels of the respective proteins. They were Bcl-2, Bad, Bax, FasL, cytochrome *c* and caspases, related to cell death and protection. Levels of mitochondrial Bad and cytosolic FasL increased in a time-dependent manner after treatment with 50 μmol/L DHA, with concomitant reduction in mitochondrial FasL (Fig. 2A). Increased expression of FasL and Bad proteins is observed after 4 h exposure to DHA. However, we could not detect any considerable change in the level of Bcl-2 or Bax protein after 24 h of exposure to DHA. Our results also revealed that DHA promoted the release of cytochrome *c* into the cytosol in E47 cells starting after 4 h of exposure, with a peak activity observed after 24 h of exposure.

Release of mitochondrial cytochrome *c* into the cytosol usually activates certain caspases, which execute apoptosis [25]. Activities of caspase 3/7 were elevated in a time-dependent manner after cells were treated with DHA for 8 h. The increased caspase 3/7 activities lasted for 24 h until they declined after 48 h, possibly due to marked cell death. (Fig. 2B) This pattern of caspase 3/7 activity is consistent with the release of mitochondrial cytochrome *c* into the cytosol in the DHA-treated E47 cells.

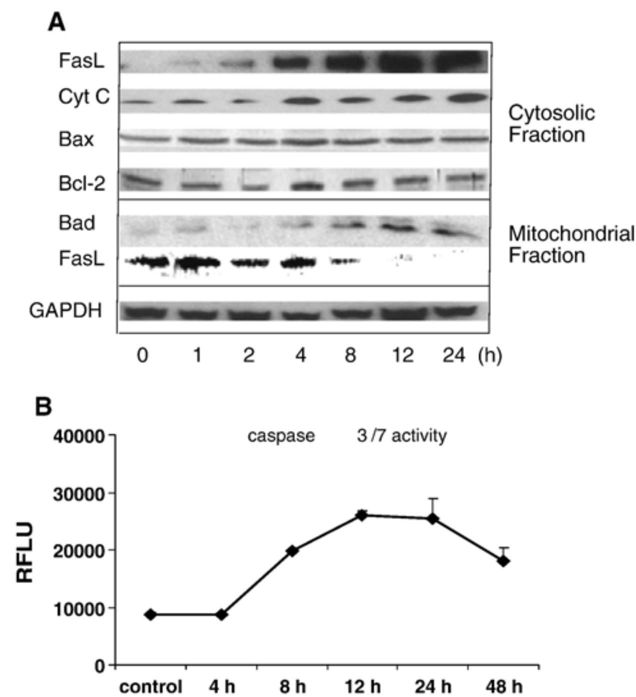

Fig. 2. DHA-induced changes in apoptosis-related proteins and caspase 3/7 activity. (A) FasL protein and mitochondrial cytochrome *c* were released into cytosol. FasL was reduced in mitochondrial fraction. Bcl-2 and Bax were not changed. (B) The changes in caspase 3/7 activity after DHA treatment (50 μmol/L).

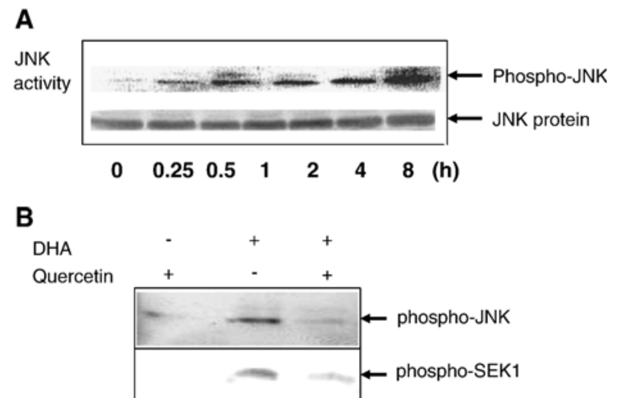

Fig. 3. Changes of JNK and SEK-1 activities in DHA-treated E47 cells. (A) JNK activated from 0.25 to 8 h by DHA treatment was changed. Total JNK protein was not changed. (B) The effects of pretreatment with the JNK inhibitor quercetin (40 μmol/L). The result of immunoblot analysis for phospho-JNK and phospho-SEK-1 showed suppression of JNK and SEK-1 by quercetin at 2 h after DHA exposure.

3.3. The changes in MAP kinase activities

MAP kinases involved in early cell signaling are known to be important in the cell survival or cell death pathways. Therefore, we investigated time-dependent changes in the activity of the respective MAP kinases involved in early signal transduction: JNK, p38 MAP kinase and ERK. Uniform levels of the target MAP kinase proteins and their respective substrate proteins during the assay were verified. Under this condition, DHA rapidly elevated JNK phosphorylation within 15 min, and the elevated level of JNK persisted up to 8 h (Fig. 3A). However, the activities of ERK and p38 MAP kinase remained unchanged throughout the DHA treatment up to 24 h in this study (data not shown). This JNK activation was followed by an increase in proapoptotic Bad and FasL starting at 4 h and was accompanied by cytochrome *c* release and increased caspase 3 activity observed at 8 h in a successive manner.

Our results suggest an important role of JNK activation in DHA-induced cell damage. Therefore, we studied the effect of a JNK inhibitor, quercetin (40 μmol/L), on JNK activity and DHA-mediated cell death rate. Quercetin efficiently reduced DHA-induced activation of JNK or its upstream kinase, SAPK (SEK-1), determined at 1 and 2 h after DHA exposure (Fig. 3B).

3.4. Role of CYP2E1 in DHA-induced cell damage

We hypothesized that CYP2E1 was involved in DHA-mediated cell damage since our current results suggest that DHA caused more damage to E47 cells than C34 cells without CYP2E1. LPOs are believed to play an important role in E47 cell damage because CYP2E1 is known to increase the levels of reactive oxygen species (ROS) that usually lead to increased LPO production [12,15]. There-

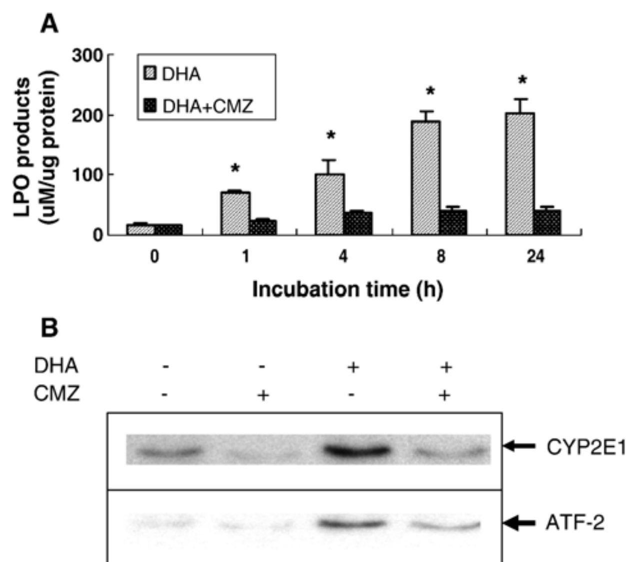

Fig. 4. Effect of 20 μmol/L CMZ, a CYP2E1 inhibitor, on LPO production, CYP2E1 induction and JNK activity in DHA-induced E47 cells. (A) Inhibitory effect of CMZ on LPO production in E47 cell (*P<.01). (B) E47 cells were pretreated with CMZ for 6 h before cells were exposed to 50 μmol/L DHA for 2 h. CMZ simultaneously inhibited CYP2E1 induction and JNK activation at 2 h, and these effects were sustained up to 24 h. Activation transcription factor-2 (ATF-2) was one of the substrates for JNK.

fore, we studied the effect of CMZ, a CYP2E1 inhibitor, on the LPO production as well as JNK activity. LPO level significantly increased in E47 cells after 8 and 24 h of exposure, and LPO was also strongly inhibited by CMZ, up to 24 h of incubation (P<.01, Fig. 4A). These results supported a role of CYP2E1 in increasing LPO production after DHA exposure and suggested that LPO might be involved in DHA-mediated cell death. Since the CYP2E1 level was increased in a time-dependent manner after treatment with 50 μM DHA in our study, LPO level corresponded with that of CYP2E1 as in other researches.

We then studied the effect of CMZ on the CYP2E1 induction and JNK activities after exposure to DHA. CMZ (20 μmol/L) that was pretreated with DHA for 6 h significantly reduced the DHA-mediated increase in CYP2E1 content in E47 cells. In addition, CMZ pretreatment significantly decreased the DHA-mediated increases in CYP2E1 induction and JNK activity observed at 2 h, and such effects were sustained for up to 24 h (Fig. 4B). CMZ pretreatment markedly reduced the rate of DHA-mediated cell death rate from 48% to 25% measured at 48 h (data not shown). This result suggests that DHA-induced cell damage could result from joint effects of elevated CYP2E1 and JNK-related cell death pathway.

4. Discussion

Previous studies indicate that dietary PUFA such as DHA can trigger apoptosis of various cancer cells, although the cell death depends on the cell type, concentration and the duration of treatment. Our results revealed that the DHA-mediated cell death of E47 cells took place mainly via apoptosis, although necrosis could occur at higher levels of DHA. Chen et al. reported that AA efficiently caused cell death of E9 HepG2 cells, which continuously express CYP2E1, possibly through the production of LPOs. The level of LPO was raised by about twofold (up to about 7 μmol/L) over the control cells after exposure to 30 μmol/L AA [15], but oleic acid (C18:1) did not cause cell death in either E9 or MV5 cells, which do not express CYP2E1. These results suggest that a higher degree of unsaturation in PUFA was more toxic to CYP2E1-containing cells through elevated levels of LPO and oxidative stress [15–18].

However, Kim et al. [19] reported that DHA protects neuronal cells from undergoing apoptosis because of DHA-induced caspase inhibition. Since neuronal cells do not contain an enzyme, which can be compared with CYP2E1 in E47 HepG2 cells, DHA might remain stable and be steadily incorporated into phospholipids (phosphatidylserine) of neuronal cells where it exerts its protective effect against apoptosis. In the case of E47 with transduced CYP2E1, DHA should have been efficiently metabolized, and its reactive oxidized metabolites may activate the cell death signaling.

In the case of HT-29 colon cancer cells, DHA decreased Bcl-2 level with an increased sensitivity to lipid peroxidation but did not change the levels of Bax and p53. The cell death of HT-29 appeared to be cyclooxygenase independent since indomethacin, a potent inhibitor of cyclooxygenase, did not prevent DHA-mediated cell death [20]. In contrast to the changes observed by Chen and Istfan [20], there were little changes in Bcl-2 and Bax after exposure of E47 hepatoma cells to DHA. Therefore, the effect of DHA on reducing Bcl-2 level may not be always observed in all cancer cells. Under our experimental conditions, DHA or its reactive oxidized metabolites produced by CYP2E1-mediated metabolism of DHA stimulates JNK activity for up to 8 h, which then increases the expression of proapoptotic FasL (between 4 and 24 h), leading to stimulation of cytochrome *c* release and caspase 3 activity prior to the actual cell death.

Siddiqui et al. [6] demonstrated that DHA-induced apoptosis in Jurkat cells increases the expression of Fas and secretion of FasL, which, in turn, causes Fas-receptor-mediated apoptosis. Alternatively, increased Bad expression (between 4 and 24 h) in mitochondria, a member of the proapoptotic Bcl-2 family, may be responsible for the cytochrome *c* release and caspase 3 activation leading to cell death [28,29]. Our current results also demonstrate that DHA causes E47 cell damage through JNK activation and proapoptotic upstream signals, accompanied by the elevation of FasL and Bad, cytochrome *c* release and caspase 3 activation.

The effect of DHA on the MAP kinases involved in cell death or cell survival pathways has seldom been studied, although the central role of JNK/SAPK activation in cell death has been generally accepted [8,9] with a few exceptions [24]. Since this is particularly true in hepatoma cells, we used HepG2 cells as a model to study the changes in MAP kinases and their roles in DHA-mediated cell

damage [30]. Our preliminary study revealed that CYP2E1 substrates such as HNE, acetaminophen and CCl_4 cause cell damage through selective activation of the JNK-related cell death pathway without activating another MAP kinase, p38 MAP kinase [24]. Pretreatment with the p38 kinase inhibitor SB203580 did not significantly change the rate of DHA-induced E47 cell death. These results suggest that p38 kinase and JNK may play different roles in DHA-induced E47 cell death. The absence of p38 MAP kinase activation via DHA is of interest because stressful conditions and several toxic agents usually increase the JNK and p38 MAP kinase activities, often in a coordinated fashion.

In contrast, pretreatment with quercetin, an inhibitor of JNK, significantly reduced JNK and its upstream kinase (SEK-1), as well as the rate of DHA-mediated cell death, supporting the critical role of JNK in cell death. Quercetin, a flavonoid molecule ubiquitous in nature, functions not only as an antioxidant but also as an inhibitor of tyrosine/serine/threonine kinase such as PKC and MAP kinase. Quercetin strongly reduced the activation of phosphorylated ERK, p38 MAP and JNK MAP kinase with its suppressing properties of No/iNOS and NF-kB signal transduction pathways in macrophages [31]. The specific inhibitor of JNK, SP600125, was not stronger than quercetin in E47 cells. However, future studies must test the effect of other typical antioxidants, alpha-tocopherol, vitamin C and vitamin E, on JNK activation.

This JNK activation may be linked to cell death signals such as FasL or Bad up-regulation, as previously suggested in certain cells such as ovarian carcinoma cells and Chinese Hamster V79 cells [32,33]. Alternatively, JNK activation may be linked to Ca^{2+} change during the DHA-mediated cell death, as indicated in ovarian carcinoma cells [32]. After pretreatment with BAPTA-AM, an intracellular Ca^{2+} chelator, the cell death rate in E47 cells was significantly reduced, similar to the baseline, until 24 h after DHA exposure. The important role of Ca^{2+} in DHA-induced apoptosis is in agreement with the involvement of Ca^{2+} in AA-induced damage of E47 cells, as recently reported [34].

This study provides another example of the critically important role of JNK activation in cell damage caused by DHA, another substrate of CYP2E1. This study was consistent with the selective activation of the SEK-1–JNK pathway by other CYP2E1 substrates such as 4-HNE [24], acetaminophen [22] or CCl_4 [35]. Our results showed that DHA caused less damage to C34 control HepG2 cells than E47 cells with transduced CYP2E1, indicating the important role of the CYP2E1-dependent metabolism of DHA. Since more E47 cells undergo cell death than C34 HepG2 cells, the roles of cyclooxygenase, lipoxygenase and other enzymes involved in the DHA metabolism may not be as important as CYP2E1 in the DHA-induced apoptosis. Consistent with this hypothesis, a significant role of cyclooxygenase and lipoxygenase could be eliminated due to the minimal effect of indomethacin, ETYA and inhibitors of lipoxygenase on the rate of DHA-induced cell death measured at 48 h (data not shown). Therefore, the critical role of CYP2E1-mediated DHA metabolism in cell death can be supported by the role of CYP2E1 in the metabolism of long-chain PUFAs and the protective effect of CMZ, an inhibitor of CYP2E1, in DHA-mediated cell damage [26]. These scenarios are supported by the result of pretreatment with CMZ, which markedly inhibited the levels of CYP2E1 and blocked the JNK activation upon exposure to DHA. Furthermore, CMZ significantly reduced the rate of DHA-induced cell death determined at 12, 24 and 48 h, supporting the critical role of JNK activation in the DHA-mediated apoptosis (data not shown).

Many cancer cells were killed by the ROS, although the killing mechanisms depended on different cell types. Higher concentration of PUFAs increases the sensitivity to ROS in tumor cells because of the cytotoxicity of LPO and its metabolites [18,36,37]. Two possible mechanisms for LPO-induced cell damage through CYP2E1-mediated metabolism of dietary fatty acids were suggested. Firstly, CYP2E1 may directly oxidize PUFAs (omega-1 hydroxylation) to reactive radicals that produce the toxicity. Secondly, ROS such as superoxide or H_2O_2 may be generated during the CYP2E1 catalytic cycle and produce LPOs such as MDA and 4-HNE when the cells are treated with PUFAs [15,27]. Our research provided the interesting finding that early signaling, such as JNK activation followed by up-regulation of proapoptotic FasL or Bad protein, may cooperate with the elevated LPO levels on DHA-mediated cell death. LPO accumulation started on the first hour and reached 50% of the maximum by 4 h after observing it for 8 or 24 h. Since the cytochrome *c* release and caspase 3/7 activity were increased for the first time after 4 and 8 h, respectively, LPO may trigger or interact with the JNK-caspase apoptotic pathway.

The most significant finding of this study is that DHA-mediated apoptosis in E47 cells is induced through the mitochondrial injury sequenced apoptosis pathway, which may be associated with the LPO levels during the CYP2E1 catalytic system.

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