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Original Article

The Inhibitory Effect of Dibutyryl Cyclic AMP on Docosahexaenoic Acid-Induced Apoptosis in HL-60 Cells through Activation of the Phosphatidylinositol-3 Kinase Pathway

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Abstract

Objective: Docosahexaenoic acid (DHA) is known as a chemopreventive substance for cancers. Previously we reported that DHA induces apoptosis in HL-60 cells. The aim of this study was to clarify the role of phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling during DHA-induced apoptosis in HL-60 cells.

Methods: The inhibitory effects of dibutyryl cAMP (db-cAMP) or LY294002 (a specific inhibitor of the PI3-kinase/Akt pathway) on DHA-induced apoptosis in HL-60 cells were evaluated by the appearance of apoptosis, and from the activities of caspases (3 and 8), the phospholylation of Akt, and cleavage of Bid using DNA indexes, emzymatic measurement of fragmented substrates, and Western blotting, respectively.

Results: The pre-incubation of db-cAMP reduced the activation of caspasses (3 and 8) during the occurrence of DHA-induced apoptosis in HL-60. However, the inhibition of PI3-kinase/Akt signaling by LY294002 resulted in recovery of the caspases' activities, appearance of apoptotic cells, and cleavage of the Bid molecule when LY294002 was co-treated with db-cAMP before the occurrence of DHA-induced apoptosis in HL-60. It was also confirmed that LY294002 strongly inhibited phospholylation of Akt during db-cAMP induced-reduction of DHA-induced apoptosis in HL-60.

Conclusion: We demonstrated that DHA-induced apoptosis was sensitive to the modulation of PI3-kinase activity by treatment with db-cAMP or LY294002. These results may provide new insights into the mechanisms of the anti-cancer activity of DHA.

Key words: docosahexaenoic acid, HL-60 cells, apoptosis, phosphatidylinositol-3 kinase pathway, Akt

Introduction

Highly polyunsaturated dietary n-3 fatty acid (PUFA), i.e., eicosapentaenoic acid (EPA) (20:5) and docosahexaenoic acid (DHA) (22:6), have been found to be protective against colon cancer in epidemiological, clinical, and experimental studies (1). These PUFA are found in high concentrations in fish and marine oils. It is clear from recently published studies (2) that fish oil is protective against experimentally induced colon cancer in part by up-regulating colonocyte apoptosis. Recently we have shown that DHA can induce apoptosis in human myeloid leukemia HL-60 cells (15). Additionally, DHA protects staurosporine-induced apoptosis via the phosphatidylinositol-3 kinase (PI3-kinase) pathway in Neuro 2A cells (3). However, in other cell types, an examination of the effects of specific n-3 PUFA has demonstrated that the effect of EPA is related mainly to its inhibitory action on cell proliferation, whereas the effect of DHA corresponds with its induction of apoptosis (4). The mechanism(s) by which DHA promotes apoptosis remain to be elucidated.

In several cells, activation of the lipid kinase, PI3-kinase, is necessary to prevent apoptosis (5, 6). There are several known downstream substrates for PI3-kinase, and preliminary studies

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indicate that activation of the serine/threonine protein kinase, protein kinase B (also known as Akt), is necessary for the observed survival effects of PI3-kinase (7). A previous study has shown that the cytoprotective effect of cAMP against bile acid-induced apoptosis may be linked to the modulation of the PI3-kinase signaling pathway in cultured rat hepatocytes (8).

In several cell types, cyclic adenosine monophosphate (cAMP) protects against apoptosis induced by death receptors or intracellular stress (9–11). However, in human cancer cells such as HL-60 leukeimic cells and SHSY5Y neuroblastoma cells, a cAMP analogue, 8-Cl-cAMP, induces apoptosis in a cell cycle-specific manner (12). The molecular basis for cAMP's survival effect in apoptosis has not been fully characterized. One study has implicated mitogen- and stress-activated kinases in the cytoprotective effect (13). In the present study, we determined whether cAMP can modulate DHA-induced apoptosis. We report that cAMP is cytoprotective against DHA-induced apoptosis and that this effect may be linked to the PI3-kinase/Akt pathway in a human myeloid leukemia cell line HL-60 cells, which have been widely investigated as a model of apoptosis.

Materials and Methods

Materials

Acetyl-Ile-Glu-Thr-Asp 4-methyl-cumaryl-7-amide (Ac-IETD-MCA) and acetyl-Asp-Glu-Val-Asp 4-methyl-cumaryl-7-amide (Ac-DEVD-MCA) were obtained from Peptide Institute, Inc. (Osaka, Japan). DHA, ribonuclease (RNase A), propidium iodide, dibutyryl cAMP (db-cAMP), LY294002 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibody against Akt and monoclonal antibody against phospho-Akt (Ser473) were purchased from Cell Signaling Technology, Inc. (Plymouth Meeting, PA, USA). BID polyclonal antibody as anti-Bid antibody and Actin monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Protein A-Sepharose was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cell line used in this study

A human leukemic cell line established at 1979 (23), HL-60, which has been used widely for various investigations into apoptosis, cell differentiation, and cancer progression, was used in this study and was maintained in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (Sigma Chemical Co.). Cells were grown in a humidified incubation at 37°C under 5% CO_2. Cells were used for assays during the exponential phase of growth. Cell viability was assessed by the exclusion of trypan blue dye.

Assay for caspase activity

HL-60 cells treated with reagents for the required times were washed with phosphate-buffered saline (PBS), and lysed with ice-cold buffer (25 mM Hepes buffer (pH 7.5) containing 10% sucrose, 0.1% CHAPS, 100 mM dithiothreitol). After disruption of cells using a sonicator, the activities of caspase-3 or 8 enzyme were determined using a 5 μM Ac-DEVD-MCA or Ac-IETD-MCA for caspase-3 and -8, respectively. The fluorescence of released 7-amino-4-methyl-coumarin (AMC) was measured using a fluorospectrophotometer. The wavelengths for excitation and emission were 380 and 460 nm, respectively (14).

Immunoprecipitation

HL-60 cells treated with reagents for the required times were washed with PBS, and lysed with ice-cold lysis buffer (1% Triton X-100, 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.2), 1 mM EDTA, 20 mM NaF, 100 mM NaCl, 1 mM Na_3VO_4) supplemented with the protease inhibitors (1 mM benzylsulfonyl fluoride and 25 μg/ml leupeptin). After disruption using a sonicator, nuclei and unbroken cells were separated by centrifugation at 15,000×*g* for 10 min. The supernatants were precleared with protein A-Sepharose. The resultant supernatants were incubated overnight with anti-Akt polyclonal antibody, followed by the addition of protein A-Sepharose, and further incubated for 2 h. The immune complexes were recovered by centrifugation, washed with lysis buffer and heated for 5 min at 100°C in a SDS-PAGE sample buffer.

Immunoblotting

The proteins were resolved on 8% SDS-PAGE and then electronic-transferred to PVDF membranes. The membranes were blocked with 1% bovine serum albumin in PBS. After extensive washing with PBS containing 0.4% Tween 20, the immunoblots were incubated with anti-phspho-Akt antibody. For the detection of Bid protein, total cell lysate were resolved on 15% SDS-PAGE and immunoblots with anti-Bid antibody. Antibody binding was visualized with ECL chemiluminescence reaction reagents (Amersham Pharmacia Biotech).

Flow cytometrical analysis

HL-60 cells were treated with reagents for the required times and harvested by centrifugation at 450×*g* for 5 min. Cell pellets were washed with PBS and fixed with cold 70% ethanol at 4°C overnight. The fixed cells were resuspended in a phosphate-citrate buffer (pH 7.5) and incubated for 1 hr at room temperature. After treatment with RNase A (0.1 μg/ml) at 37°C for 1 hr, cells were stained with propidium iodide (10 μg/ml) at 4°C in the dark and analyzed on a flow cytometer with argon laser excitation at 488 nm. Fluoresence was detected through a 564–675 nm band filter (EPICS XL, BECKMAN COULTER).

Statistical analysis

All experiments were performed at least three times. The significance of the differences between experimental groups was analyzed by Fisher's parametric least significant difference test.

Results

DHA-induced apoptosis of HL-60 cells

As shown in Fig. 1 (panels C) DHA induced apoptosis in HL-60 cells. The apoptotic cells are shown as the percentage of the pre-G1 population in Fig. 1. Next, the effect of cAMP on DHA-induced apoptosis in HL-60 cells was evaluated by flow cytometry. Pretreatment for 30 minutes with 100 μM of a membrane-permeable cAMP analogue, db-cAMP, resulted in

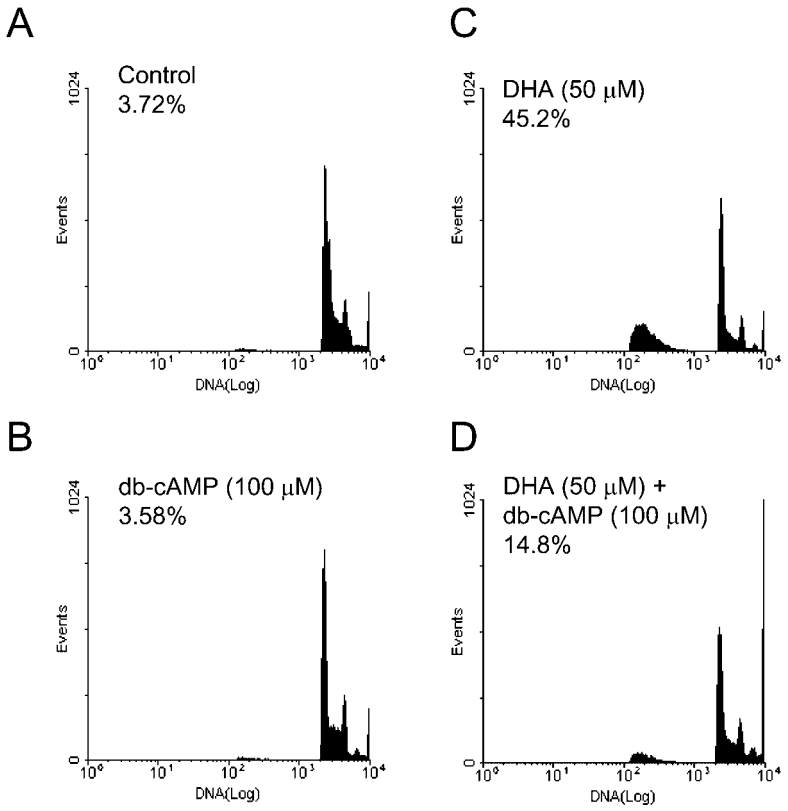

Fig. 1 Inhibition of DHA-induced apoptosis by db-cAMP in HL-60 cells. Cells were pretreated with (panel D) or without (panel C) 100 μM of db-cAMP for 30 min and then treated with 50 μM of DHA for 18 h. The cells were then stained with propidium iodide, and the pre-G1 population was determined. The apoptotic cells were shown as a percentage of the pre-G1 population. The results are representative of at least three independent experiments.

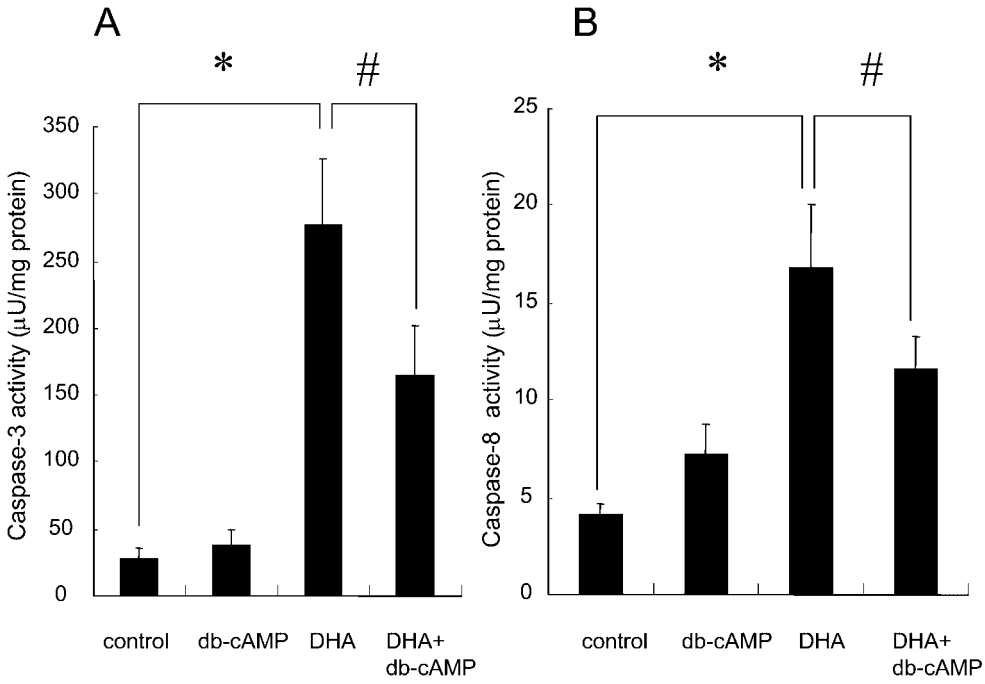

Fig. 2 Inhibition of DHA-induced caspase-3 and -8 activity by db-cAMP in HL-60 cells. HL-60 cells were pretreated with 100 μM of db-cAMP for 30 min and 50 μM of DHA was then added for 18 h. The activity of caspase-3 (panel A) or caspase-8 (panel B) was determined using 5 μM of Ac-DEVD-MCA or Ac-IETD-MCA, respectively. Results expressed as mean±SD of three experiments (* $p<0.0001$; # $p<0.005$).

protection against DHA-induced apoptosis (Fig. 1, panel D).

Effects of db-cAMP on caspase activation

Next, we evaluated the effects of cAMP on DHA-induced caspase activation. DHA-induced apoptosis was inhibited by the broad-spectrum caspase inhibitor Z-VAD-fmk (data not shown). DHA-induced apoptosis was associated with an increase in the activity of caspase-3, the final effector caspase which is involved in most apoptotic pathways. In addition, the activity of caspase-8, an initiator in the apoptotic signaling machinery, was also increased during DHA-induced apoptosis. Pretreatment for 30 minutes with 100 μM of db-cAMP inhibited DHA-induced increases in caspase-3 and caspase-8 activity (Fig. 2, panels A and B, respectively).

Inhibition of PI3-kinase/Akt signaling by DHA

We investigated whether DHA can inhibit PI3-kinase/Akt signaling. In order to confirm the involvement of the PI3-kinase pathway in DHA-induced apoptosis, we investigated the phosphorylation of Akt, a downstream signaling molecule of the PI3-kinase pathway, and the effect of LY294002, a specific PI3-kinase inhibitor, on the phosphorylation of Akt. As shown in Fig. 3, the level of phosphorylated Akt protein was significantly decreased in response to co-treatment of LY294002 and DHA. Pretreatment with db-cAMP partly canceled the inhibitory effect of LY294002. Total Akt protein levels remained constant during drug treatment (Fig. 3).

Activation of caspase-8 by DHA with PI3-kinase/Akt inhibitor

Next we investigated whether PI3-kinase/Akt inhibition occurs at the initial phases of DHA-induced apoptotic signaling. As shown in Fig. 4A, although treatment of the cells with DHA increased caspase-8 activity, pretreatment with LY294002 slightly enhanced DHA-induced caspase-8 activity. LY294002 treatment alone induced caspase-8 activity slightly (Fig. 4A). Furthermore, flow cytometric analysis showed slightly enhanced DHA-induced apoptosis by pretreatment with LY294002 for 30 min (Fig. 4B). LY294002 treatment alone did not show any

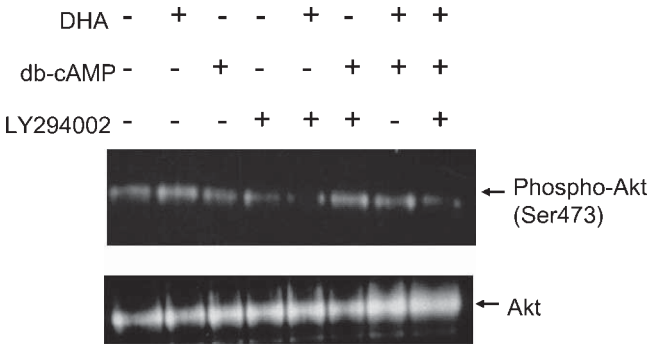

Fig. 3 Suppression of phospho-Akt content in HL-60 cells by co-treatment of DHA and LY294002. HL-60 cells were pretreated with 10 μM of LY294002 for 30 min and 50 μM of DHA or 100 μM of db-cAMP was then added for 8 h. Phospho-Akt or Akt was detected by immunoprecipitation with anti-Akt polyclonal antibody and western blot analysis with anti-phospho-Akt monoclonal antibody or Akt polyclonal antibody. The results are representative of at least three independent experiments.

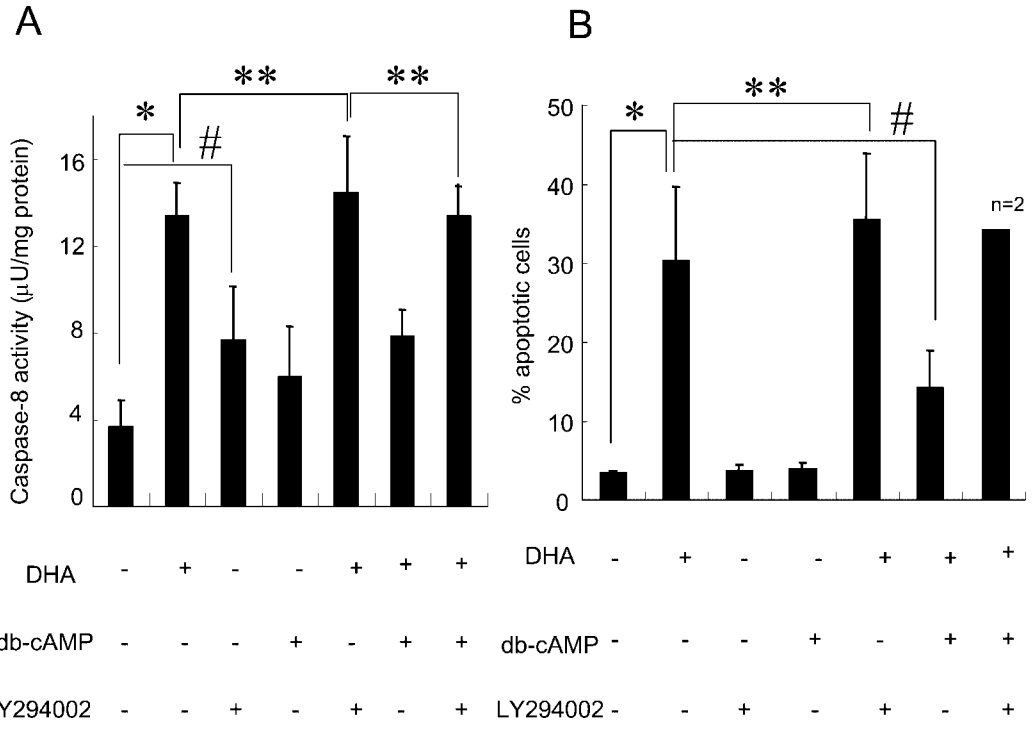

Fig. 4 Effect of LY294002 on DHA-induced caspase-8 activity and apoptosis in HL-60 cells. (Panel A) HL-60 cells were pretreated with 10 μM of LY294002 for 30 min and 50 μM of DHA or 100 μM of db-cAMP was then added for 18 h. The activation of caspase-8 was determined with 5 μM of Ac-IETD-MCA. (Panel B) Cells were pretreated with 10 μM of LY294002 for 30 min and then 50 μM of DHA or 100 μM of db-cAMP was added for 18 h. The cells were then stained with propidium iodide, and the pre-G1 population was determined. Results expressed as mean±SD of three experiments (* $p<0.0001$; # $p<0.005$; ** $p<0.05$).

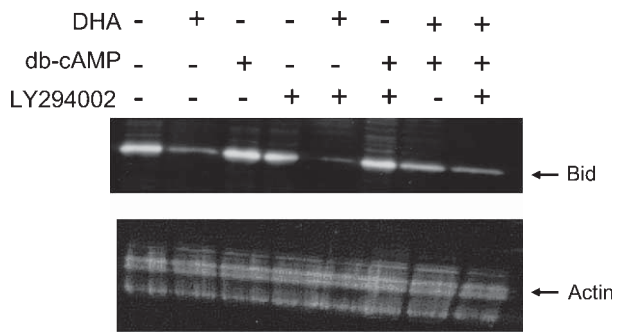

Fig. 5 Effect of LY294002 on DHA-induced Bid cleavage in HL-60 cells. Cells were pretreated with 10 μM of LY294002 for 30 min and 50 μM of DHA or 100 μM of db-cAMP was then added for 18 h. Cell lysates (40 μg) were analyzed by Western blot analysis with Bid polyclonal antibody. The result is representative of at least three independent experiments.

apoptosis inducing effect (Fig. 4B). Moreover, pretreatment with LY294002 partly canceled the preventive effect of db-cAMP. These data suggest that the DHA-induced apoptotic pathway is related to the PI3-kinase/Akt pathway.

Decrease in full-length Bid by DHA with PI3-kinase inhibitor

We studied the effect of DHA on the cellular levels of full-length Bid. Western blot analysis revealed that Bid was present as 23 kDa protein in intact HL-60 cells (Fig. 5). Treatment of cells with DHA caused a decrease in the amount of 23 kDa protein (Fig. 5). Pretreatment with 10 μM of LY294002 for 30 min enhanced the decrease in the amount of full-length Bid (Fig. 5). db-cAMP treatment alone did not induce the decrease of full-length Bid (Fig. 5). These results indicate that DHA-induced caspase-8 activation and Bid cleavage are related to the PI3-kinase/Akt pathway.

Discussion

In this study, we showed that DHA-induced apoptosis is protected by treatment with a membrane-permeable cAMP analogue, db-cAMP, and that the DHA-induced apoptotic pathway may be related to the PI3-kinase/Akt pathway. In addition, we investigated the activity of caspase-8 and the amount of Bid protein in HL-60 cells. A previous study has suggested that Bid cleaved by caspase-8 directly triggers the release of cytochrome c from mitochondria (17). No study has indicated the relationship between DHA and cAMP on the apoptotic pathway in a cancer cell line such as HL-60 cells, although recent studies have shown the influence of cAMP on the regulation of DHA release in rat brains (18) and on the antihypertensive effect of DHA in spontaneously hypertensive rats (19).

The findings of the present study demonstrated that db-cAMP inhibited DHA-induced caspase-8 and -3 activation, decrease of full-length Bid protein, and apoptosis in HL-60 cells. In regard to the mechanism by which cAMP activates PI3-kinase/Akt pathway, Cynthia et al. have previously shown that cAMP protects cultured-rat hepatocytes against glycochenodeoxycholate-induced apoptosis and that this protection is PI3-kinase-dependent (8). Furthermore, Zhang et al. have shown that PI3-kinase/Akt signaling attenuated caspase-8 activity in TNF-α-treated bovine carotid artery endothelial cells (16).

The antiapoptotic effect of Akt, a downstream effector of the PI3-kinase pathway, has been well documented in both *in vitro* and *in vivo* studies (3). Phosphorylated Akt phosphorylates Bad, a pro-apoptotic member of the Bcl-2 family, and inactivates its apoptotic function (20). In the present study, we have not evaluated the phosphorylation status of Bad in HL-60 cells. Co-treatment by LY294002, a specific PI3-kinase inhibitor, and DHA strongly induced the decrease of full-length Bid and the reduction of the Akt phosphorylation. Moreover, DHA-induced caspase-8 and -3 activation and the subsequent apoptosis were slightly enhanced by the treatment of LY294002. Zhang et al. have shown that PI3-kinase/Akt signaling attenuated caspase-8 activity in TNF-α-treated bovine carotid artery endothelial cells (16). These data indicate that the inhibition of the phosphorylation of Akt induced by DHA and LY294002 might act as an initial step in the apoptosis pathway, and that the PI3-kinase/Akt pathway is probably related to the DHA-induced apoptotic pathway. Furthermore, the recent studies suggest that PI3-kinase activates production of Bcl-2 (21), which then inhibits ceramide production (22). We cannot exclude the possibility that DHA-induced apoptosis is related to the ceramide production. Further investigation is required to define the DHA-induced apoptotic mechanisms.

In conclusion, we showed that DHA-induced apoptosis was sensitive to the modulation of PI3-kinase activity by treatment with db-cAMP or LY294002. In this study, we showed that DHA-induced apoptosis was inhibited by pretreatment with db-cAMP, but that this inhibition was partial. Since the mitogen-activated protein (MAP) kinase pathway has been implicated in transducing survival signaling as well as PI3-kinase/Akt pathway in a wide range of cell types, we need to investigate the involvement of this pathway in DHA-induced apoptosis signaling. Although the relationship between the DHA-mediated apoptotic pathway and the PI3-kinase/Akt pathway requires further study, these results may provide new insights into the mechanisms of the anti-cancer activity of DHA.

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