Induction of apoptosis in pheochromocytoma (PC12) cells exposed to eicosapentaenoic acid in vitro

Mei Li, Haitao Ge, Xiuqin Kong, Weifa Zheng1 and Zhili Liu*

Department of Biology, College of Life Sciences, Nanjing University, Nanjing, 210093, Jiangsu, China
1Key Laboratory of Biotechnology for Medicinal Plants of Jiangsu Province, Xuzhou, 221116, Jiangsu, China

Received 5 November 2004; revised 29 June 2005

The effect of different concentrations of eicosapentae noic acid (EPA), on rat pheochromocytoma PC12 cells were evaluated using cell viability, lactate dehydrogenase (LDH) activity, flow cytometric DNA analysis and electronic microscopy. A time- and dose-dependent decrease in the cell viability was observed in the cultures of PC12 cells, supplemented with EPA. The incubation with 200 µM EPA for 48 and 72 hr induced a decrease in the cell viability by 53.40 and 53.43%, respectively. Treatment of PC12 cells with EPA induced apoptosis in a concentration-dependent manner, as evidenced by flow cytometry analysis. The highest percentage of apoptotic cells accumulated to 30.32%, following treatment with 200 µM EPA. The LDH levels increased significantly on treatment with 100 and 200 µM EPA, by 144.4 and 197.3%, respectively, compared with the untreated control. In addition, the cell morphology change was also observed by electron microscopy. The results suggest that EPA mediates its effect on the PC12 cells, at least in part, via the induction by apoptosis.

Keywords: Eicosapentaenoic acid, pheochromocytoma PC12, cell viability, lactate dehydrogenase, flow cytometric DNA analysis, electronic microscopy, apoptosis

IPC Code: G01 N 33/574, 901 J 37/26

5, 8, 11, 14, 17-Eicosapentaenoic acid (EPA), one of the major polyunsaturated fatty acids (PUFAs) is found almost exclusively in the aquatic animals. It is synthesized by phytoplankton, which is then consumed by fish, mollusks and crustaceans, and thereby is concentrated in the aquatic food chain1. Dietary long-chain PUFAs have been suggested to play a significant role in modulating cancer development2-3. The exogenous fatty acids are reported to modulate the cytotoxic activity of several anticancer drugs in cell culture4 and in tumor-bearing animals5. For example, the human breast cancer cells in vitro6 or growing in athymic nude mice as solid tumor7 support the inhibitory effects of n-3 PUFAs, such as EPA and docosahexaenoic acid (DHA). An imbalance in cell proliferation and cell death leads to tumour growth. n-3 PUFAs may affect the cancer cells, by evoking the apoptosis (term used to describe the peculiar morphology of programmed cell death) of cancer cells5.

Earlier, ability of EPA to induce apoptosis in vitro in human leukemic and pancreatic cancer cells has been demonstrated9-11. These studies suggested potential involvement of apoptosis in controlling cancer cell growth with EPA. In the present paper, we investigated the effect of EPA on pheochromocytoma (PC12) cells, and studied the possible mechanisms using different techniques to indicate that EPA induces cells death by induction of apoptosis.

Materials and Methods

EPA (Sigma, purity > 99%) was dissolved in ethanol and stored in the dark as stock solution at -20°C. The different concentrations of EPA were freshly prepared from the stock solution and diluted with growth medium.

Cell culture

The PC12 cell line was obtained from Shanghai Institute of Cell Biology and grown on plastic without polylsine or collagen coating. The cells were routinely maintained in RPMI 1640 medium (Gibco),
supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, sodium bicarbonate and HEPES (Sigma). Culture was carried out at 37°C in 95% humidified air with 5% CO₂ incubator. These cells were seeded on 6-, 24- and 96 wells flat-bottom plates at a density of approx. 5 × 10⁵ cells/ml in the medium described above and exposed to EPA for 24, 48 and 72 hr.

Morphological evaluation

The morphology of PC12 cells was examined and photographed after the exposure to different concentrations of EPA. The cells were stained with classic hematoxylin-eosin (H.E) and photographed using a Nikon T1200 microscope equipped with Jieda-801 morphologic analysis system (Jieda Tec. Co. Ltd., Jiangsu).

MTT assay

After 24, 48 or 72 hr exposure to EPA, cell survival was determined by a standard colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT was dissolved in phosphate buffer saline (PBS) at 5 mg/ml. From this stock, 20 µl per 100 µl of medium was added to each well and incubated for 4 hr at 37°C. The medium was removed and 200 µl of dimethylsulfoxide (DMSO) was added per well and plates were incubated overnight at 37°C (avoiding light) to dissolve the blue formazan product. Absorbance was measured at 570 nm in an automated plate reader (Sunrise Co. Ltd) using the readings of DMSO-treated wells as blank. Growth inhibition was expressed as a percentage of the DMSO-treated controls that were processed simultaneously, using the following equation: (A₅₇₀ of EPA-treated sample – A₅₇₀ of DMSO-treated)/(A₅₇₀ of untreated – A₅₇₀ of DMSO-treated) × 100%.

Flow cytometry

An index of apoptosis was obtained from measurements of DNA content of individual cells using flow cytometry. PC12 cells were seeded on to 6-well plates and treated with 10, 30, 50, 100 and 200 µM EPA, respectively for 72 hr, to determine the concentration-response relationship. The adhered and floating cells were mixed and washed with cold PBS twice, centrifuged (1000 × g, 10 min), resuspended in 0.5 ml 70% cold ethanol and stored overnight at 4°C. After centrifugation (1000 × g, 10 min) to remove the ethanol, and washing again with cold PBS, the cells were resuspended in PBS containing RNAase A 100 µg/ml and incubated at 37°C for 30 min, centrifuged again at 1000 × g for 10 min. The cells were then stained with PBS (pH 7.4) containing propidium iodide (PI) 50 µg/ml at 4°C for 30 min (avoiding light). Flow cytometry was performed using a Becton-Dickenson FACScan flow cytometer and Cell Quest software version 1.2 (Becton-Dickenson, Mountain View, CA, USA) to calculate the apoptosis percentage.

Determination of lactate dehydrogenase (LDH) activity

Cytotoxicity was determined by estimation of LDH activity in the supernatant. After incubation for 72 hr, the cells were centrifuged at 1000 × g for 5 min and the supernatant was kept for LDH determination. Sedimented cells were washed twice by resuspending in PBS and recovered by centrifugation at 1000 × g for 5 min. The cell pellet was resuspended in PBS (pH 7.4), frozen and defrosted three times, and centrifuged at 1000 × g for 10 min to remove any non-adherent cells. LDH activity was determined spectrophotometrically at 340 nm as described.

Electron microscopy

PC12 cells treated with 100 and 200 µM EPA for 72 hr were collected and washed in PBS, fixed for 5 min at 4°C with 2% paraformaldehyde in PBS (pH 7.4), post-fixed in 2% osmium tetroxide (OsO₄), dehydrated with a graded series of acetone and embedded in Luveak-812 (Nacalai Tesque, Kyoto, Japan). Ultrathin sections were stained with lead citrate and uranyl acetate, and examined with a Hitachi 600-II electron microscopy (Hitachi, Tokyo).

Data analysis

All experiments were repeated, and the data were expressed as mean ± S.D. Statistical significance was determined by the one-way analysis of variance and covariance (ANOVA), followed by Tukey’s pair-wise comparisons at significance level of 0.05.

Results

Effect of EPA on the morphology of PC12 cells

The morphology of untreated control PC12 cells after 4 hr of inoculation turned from rotundity into multi-angled and strip shapes gradually. After 48 hr, the rounded cells number decreased, and cell protuberance grew thicker and bigger. The PC 12 cells treated with 10, 30 and 50 µM EPA grew well, but not the 100 and 200 µM groups. Compared with
the untreated control cultures, EPA at concentrations higher than 100 µM induced the death of the majority of cells. The cells were mostly round and the adhesion was not quite firm at 200 µM EPA. The PC12 cells incubated for 72 hr, without and with different EPA concentrations are shown in Fig. 1.

Effect of EPA on viability of PC12 cells in vitro

Fig. 2 shows the viability of PC12 cells in the presence of various EPA concentrations at 24, 48 and 72 hr. The cell viability decreased in time- and dose-dependent manner; it decreased both with the increase in culture period and EPA concentration. Incubation for 48 and 72 hr with EPA induced a concentration-dependent cell death. Significant reduction was observed at 100 (P<0.01) and 200 µM (P<0.001) EPA concentrations. At 200 µM EPA, the cell viability decreased to 53.40 and 53.43%, for 48 and 72 hr incubation respectively, compared to control. Incubation with 10 and 30 µM of EPA for 24 or 48 hr did not cause any significant reduction in cell number.

Effect of EPA on LDH levels of PC12 cells

After exposure to various concentrations of EPA for 72 hr, the LDH content of PC12 cells was calculated. LDH content increased with the increase of EPA concentration. In 100 and 200 µM EPA-treated cultures, LDH levels were increased significantly by 144.4 and 197.3%, respectively, compared to the control (P<0.001) (Fig. 3). However, no significant increase was observed in 10, 30 and 50 µM EPA-treated cultures.

Effect of EPA-induced apoptosis in PC12 cells

The sub-G1 peak in flow cytometry detection is considered as indication of the apoptosis of cells. Although in untreated cells, the peak was undetectable (Fig. 4 A), the treatment with different concentrations of EPA produced sub-G1 peak (Fig. 4 B-F). The percentage of apoptotic cell accumulation in the sub-G1 peak was calculated by flow cytometry analysis. EPA induced the apoptosis in a concentration-dependent manner (Fig. 5). The 50, 100 and 200 µM EPA significantly increased the apoptotic
cells accumulation in the sub-G₁ peak (P<0.01); the highest accumulation was 30.32%, following the treatment with 200 µM EPA. No significant increase was observed with 10 and 30 µM EPA.

**Effect of EPA on ultrastructure of PC12 cells**

PC12 cells showed typical morphological characteristics of apoptosis in different stages after exposure to 200 µM EPA for 72 hr (Fig. 6). In early stage, the chromatin was fragmented and accumulated to the inside of nucleolus membrane. In the final stage, the cell membrane wrapped up the fragmented chromatin, indicating the induction of cell apoptosis.

**Discussion**

There is a considerable interest in exploiting the dietary effects of fatty acids in cancer prevention, and also as an adjunct to conventional cancer therapy. Certain PUFAs, particularly those found in fish oil and microalgae have beneficial dietary effects in a variety of health problems, such as cardiovascular diseases, and could inhibit the proliferation of human leukemic cells and kill them in vitro⁸,¹⁴. EPA is reported to inhibit the growth of the pancreatic cancer cells by inducing cell apoptosis¹⁰. The PUFAs, including EPA and γ-linolenic acid (GLA) have been shown to induce apoptosis in a variety of cell types in vitro⁸,¹⁴. The present study demonstrated the potential tumoricidal activity of EPA on PC12 cells. EPA was selected, as it is an effective anticancer fatty acid with negligible side-effects¹⁵.

Similar to previous reports⁹, EPA in the present study showed inhibitory activity against PC12 cells viability. PC12 cells treated with 50, 100 and 200 µM EPA could effectively decrease the survival rate in a time- and dose-dependent manner; the 200 µM EPA in 72 hr reduced the cell viability to 53.43%, as compared to the control. Earlier, Chiu et al.¹⁶ and Gillis et al.¹¹ reported that the cell viability of the HL-60 cells treated with 100 µM EPA for 24 hr were reduced to 44% and 27%, respectively. Based on previous reports¹⁷,¹⁸, it can be suggested that the anti-tumor effect of a given fatty acid depends on the type of tumor cell used.
Apoptosis is a mode of programmed cell death, and is an important biological consequence of exposure to extrinsic agents. Flow cytometry has been used as one of the effective techniques to investigate the apoptosis of cells. Cell shrinkage and formation of apoptotic bodies, DNA fragmentation due to activation of endogenous endonucleases, and appearance of a sub G1 fraction in flow cytometry are the characteristic features of apoptosis. In EPA-treated PC12 cells, apoptosis was confirmed by the appearance of a sub G1 fraction and distinctive morphological changes. The apoptosis in EPA-treated cells was induced in a concentration-dependent manner as evidenced by flow cytometry analysis. The morphological change of apoptotic PC12 cells was also evident in the electronic images.

Several mechanisms, including modulation of membrane properties, eicosanoid formation and free radical generation have been postulated to account for the anticancer activities of PUFAs. Stimulation of lipid peroxidation is also suggested to result in apoptosis; reduced level of lipid peroxidation was reported in hepatic tumors. In the present experiment also, higher levels of lipid peroxidation product malonaldehyde (MDA) were observed in the cells treated with higher EPA concentrations; however, no significant change in MDA content of the medium (without cells) was observed with different concentrations of EPA (data not shown). As a result of these changes, there could be a change in the status of the cells from a balanced pro/anti-oxidant to a predominant pro-oxidant state. Such a change could damage the cell membrane and DNA, leading to the death of the tumor cells. Thus, in PC12 cells, the mechanism of apoptosis, induced by EPA may be due to lipid peroxidation. The conjugated EPA and DHA have been reported to induce apoptosis via lipid peroxidation in cultured human tumor cells.

The cytotoxicity of PUFAs could also be due to the surfactant nature of their molecules. As PUFAs interact with cellular membranes, they might directly affect the stability and functions of these membranes and modulate membrane-bound enzymes and/or phospholipids, thereby triggering the apoptosis. Some reports suggest that apoptosis is caused by suppression of arachidonic acid (AA) metabolism. EPA is a competitive inhibitor of AA metabolism, and could possibly inhibit the tumor growth by decreasing the formation of prostaglandins. Some activities of n-3 fatty acids are clearly linked to the modulation of cytokine activity, and in particular that of tumor necrosis factor (TNF) in cancer cachexia. But, the detailed mechanism of EPA-induced apoptosis has not yet been fully understood. The results of the present study suggest that EPA mediates its effect on the PC12 cells, at least in part, via the induction by apoptosis.

Acknowledgement
The authors gratefully acknowledge Hua Zichun’s lab for the assistance provided in measurements of flow cytometry. We thank Dr Cao Dehua for his useful discussions. This work was supported by a grant from Jiangsu Post-doctoral Foundation.

References
Biol 72, 35-41