Docosahexaenoic Acid Inhibits Adipocyte Differentiation and Induces Apoptosis in 3T3-L1 Preadipocytes

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Abstract

Docosahexaenoic acid (DHA, C22:6), a (n-3) fatty acid in fish oil, has been shown to decrease body fat and fat accumulation in rodents. We investigated the direct effect of DHA on cell growth, differentiation, apoptosis, and lipolysis using 3T3-L1 adipocytes. Cells were treated with 25–200 μmol/L DHA containing 0.2 mmol/L α-tocopherol or bovine serum albumin vehicle as a control. Proliferation of preconfluent preadipocytes was not affected by the DHA treatment. When added to postconfluent preadipocytes, all concentrations of DHA inhibited differentiation-associated mitotic clonal expansion (P < 0.01). Postconfluent preadipocytes demonstrated apoptosis after 48 h with 100 μmol/L DHA and after 24 and 48 h with 200 μmol/L DHA (P < 0.01). Differentiation was examined by Oil Red O staining and glycerol-3-phosphate dehydrogenase (GPDH) activity after DHA treatment for 6 d. DHA decreased mean droplet size and percent lipid area in a dose-dependent manner (P < 0.01). GPDH activity was also decreased by DHA treatment (P < 0.01). In fully differentiated adipocytes, DHA increased basal lipolysis compared with the control (P < 0.01). These results demonstrate that DHA may exert its antiobesity effect by inhibiting differentiation to adipocytes, inducing apoptosis in postconfluent preadipocytes and promoting lipolysis.

Introduction

There has been considerable interest in the role of dietary fat in the development of adiposity. Total body fat and abdominal and epididymal fat mass were considerably reduced in rodents fed sources of (n-3) PUFA such as fish oil or perilla oil compared with lard or corn oil–fed groups (1,2). The mechanisms for the decreased fat deposition by (n-3) PUFA are not fully understood. This effect was thought to result from limited adipose tissue hypertrophy through enhancing fat mobilization and inhibiting hepatic lipogenesis (3,4) rather than lower energy intake.

Adipocytes play a central role in maintaining lipid homeostasis and energy balance by storing triacylglycerol (TG) or releasing FFA in response to changes in energy demands. Because the growth of adipocyte tissue can be due to both hyperplasia and hypertrophy of adipocytes (5), the process of adipocyte proliferation and differentiation has been the focus of several studies (5,6). Although relatively little research has been done on the effects of fatty acids on adipocyte cells, fatty acids have been shown to act as signal transducing molecules in the differentiation of adipocytes. Exposure of preadipocytes to palmitate led to a strong stimulation of cell differentiation by increasing both the postconfluent proliferation of preadipocytes and the level of expression of terminal differentiation-related genes (7). In contrast, conjugated linoleic acid inhibits differentiation (8) and reduces transcription of differentiation marker genes such as adipocyte fatty acid binding protein (aP2) and peroxisomal proliferator-activated receptor-γ in 3T3-L1 adipocytes (9). However, studies on the direct effect of (n-3) fatty acids on adipocyte development in vitro are limited.

Docosahexaenoic acid (DHA, C22:6), a (n-3) PUFA in fish oil, inhibits the proliferation not only of various cancer cells but also of normal cells (10–12). Several mechanisms have been suggested for the antiproliferative effects on tumor cells, including peroxidative damage from lipid peroxidation (11,13), apoptosis induction (14), modulation of eicosanoids (15), and increasing membrane permeability to increase susceptibility to anticancer drugs (16). Interestingly, apoptosis in adipose tissue has been described (17) and it has been suggested that adipocyte deletion by apoptosis could be a contributor to body fat loss (18).

The purpose of this study was to examine the potential of DHA to function as an antiobesity agent via modulation of adipocyte lipolysis storage and/or preadipocyte cell proliferation. It specifically explored the effects of DHA on 3T3-L1 preadipocyte differentiation, lipolysis, and apoptosis.

Materials and Methods

Materials and cell culture. 3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection and cultured as...
described elsewhere (19). Briefly, cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine calf serum until confluent. Two days after postconfluence (d 0), the cells were stimulated to differentiate with 0.5 mmol/L isobutylmethylxanthine, 1 mmol/L dexamethasone, and 167 nmol/L insulin added to DMEM containing 10% fetal bovine serum (FBS) (differentiation medium containing isobutylmethylxanthine, dexamethasone, and insulin [MDI]) for 2 d (d 2). Cells were then maintained in 10% FBS/DMEM medium with 167 nmol/mL insulin for another 2 d (d 4), followed by culturing with 10% FBS/DMEM medium until analysis. Approximately 5 d after induction of differentiation, >90% of cells displayed the characteristic lipid-filled adipocyte phenotype.

DHA (Sigma) was delivered to the cells as fatty acid/bovine serum albumin (BSA) complexes. The molar ratio of fatty acid to BSA was 4:1. DHA stock was aliquoted and stored at −20°C. All DHA treatments contained 0.2 mmol/L α-tocopherol (Sigma) to prevent lipid peroxidation. BSA vehicle without α-tocopherol was used as a control. DHA-BSA complex was added to the cultures at the concentrations and times indicated for each experiment. All medium contained 100 kU/L penicillin, 100 mg/L of streptomycin, and 292 mg/L of glutamine (Invitrogen). Cells were maintained at 37°C in a humidified 5% CO2 atmosphere.

Cell viability assay. The MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS; Celltiter 96Aqueous One solution, Promega] was performed in a time-course manner to determine the number of viable cells in culture. For preconfluent preadipocytes, cells were seeded in 96-well plates at a density of 2500 cells/well and BSA vehicle or DHA (25, 50, and 200 μmol/L) was added at 24 h after cell seeding. For postconfluent preadipocytes, DHA was added with MDI differentiation medium at d 0. Cells were incubated for 24, 48, and 72 h in both experiments. The absorbance was measured at 490 nm in a plate reader (Quant Bio Tek Instruments) to determine the formazan concentration, which is proportional to the number of living cells in culture.

Adipogenesis. Cells were seeded in 35-×10-mm culture dishes and grown to confluence. DHA (50 and 200 μmol/L) or BSA vehicle was added with the MDI differentiation medium at d 0 and cultured according to the differentiation protocol for 6 d with treatments. At d 6, cells were stained with Oil Red O and hematoxylin as described by Suryawan and Hu (20). After mounting with glycerol gelatin, 3 images for each dish were captured and analyzed for mean lipid droplet size and percentage of lipid area using ImagePro software (Mediacybernetics).

Glycerol-3-phosphate dehydrogenase activity. In addition to Oil Red O staining, glycerol-3-phosphate dehydrogenase (GPDH) assay was used as a marker of late adipocyte differentiation. Cells were seeded in a 6-well plate and grown to confluence. After 6 d of differentiation in the absence or presence of DHA (25, 50, and 200 μmol/L) (d 0–d 6), the cells were rinsed 3 times with PBS, scraped into 0.5 mL ice-cold sucrose buffer containing 0.28 mol/L sucrose, 5 mmol/L Tris, 1 mmol/L EDTA, 0.002% β-mercaptoethanol, and stored at −70°C. The homogenate was sonicated with 3 blasts for 15 s and centrifuged at 10000 × g; 10 min at 4°C and the resulting supernatants were used for GPDH assays according to Wise and Green (21). Activities are expressed in mU/mg of protein (1 mU being equal to the oxidation of 1 nmol of NADH/min). Protein was measured using the BCA protein assay kit (Pierce).

Lactate dehydrogenase activity. Cells were seeded in 96-well plates at a density of 2500 cells per well and DHA (25, 50, and 200 μmol/L) was added at 24 h after cell seeding. After 48 and 72 h of culture with DHA treatment, lactate dehydrogenase (LDH) assay was performed using CytoTox-ONE (Promega) according to the manufacturer’s instructions. LDH activity released in response to treatment and maximum LDH release by complete lysis of cells were determined with fluorescence. Data are presented as the percentage of LDH released into the medium relative to maximum LDH control. For postconfluent cells, DHA or vehicle was applied at d 0 with MDI differentiation medium and LDH release was measured after 24 and 48 h of culture.

**Apoptosis assay.** Single-stranded DNA ELISA kit was used for detection of apoptosis in 3T3-L1 cells (ApoStrand, BIOMOL). This assay is based on the selective denaturation of DNA in apoptotic cells by formamide, which reflects changes in chromatin associated with apoptosis. DHA (25, 50, 100, and 200 μmol/L) was added to 2-d postconfluent cells with MDI differentiation medium. After 24 and 48 h of culture, cells were fixed and the procedure described previously was followed (18). The absorbance was read using an ELISA plate reader at 405 nm.

**Lipolysis assay.** Fully differentiated adipocytes (d 8–10) were treated for 4 h with vehicle or DHA (50, 100, and 200 μmol/L) with or without epinephrine (1 μmol/L). The conditioned medium was removed from each well and assayed for glycerol content with Free glycerol determination kit (Sigma). The time point was chosen from a preliminary test, and it was within the linear range of glycerol release.

**Statistical analysis.** All values were expressed as means ± SEM and 1-way ANOVA was used to determine significance of treatment effects at each time point. Differences among treatment means were determined by Tukey’s test and a P-value of < 0.05 was considered significant.

**Results**

**Effect of DHA on preadipocyte and adipocyte viability.** DHA treatment of mature adipocytes did not affect cell viability (data not shown). DHA treatment did not change the number of viable preconfluent preadipocytes appreciably (Fig. 1A). LDH release by DHA treatment did not increase during preconfluent proliferation stage (data not shown). We conclude that DHA did not affect proliferation of preconfluent preadipocytes.

In contrast to the preconfluent preadipocytes, all concentrations of DHA (25, 50, and 200 μmol/L) reduced the number of viable cells compared with control during the postconfluent proliferation stage (P < 0.01, Fig 1B). Although LDH release into the culture medium increased in all cultures over time, high doses of DHA triggered greater LDH release during postconfluent mitotic clonal expansion (P < 0.05). LDH release was...
increased by >2-fold in cultures containing 50 (after 48 h) and 200 (after both 24 and 48 h) \( \mu \text{mol/L} \). DHA compared with control (Fig. 2A). This result indicates that some cytotoxic effect of DHA might play a role in the inhibitory effect on mitotic clonal expansion.

**Effect of DHA on apoptosis.** We examined whether the inhibitory effect of DHA on MDI-induced mitotic clonal expansion could be related to apoptosis. An apoptotic effect of DHA during the early stage of differentiation was evident. DHA increased apoptosis in the postconfluent preadipocytes in a time- and dose-dependent manner with considerable effects observed after 24 and 48 h with 200 \( \mu \text{mol/L} \) DHA and after 48 h with 100 \( \mu \text{mol/L} \) DHA (\( P < 0.01 \), Fig. 2B). This result indicates that the decrease in viable postconfluent cells is due at least in part to the effect of DHA on inducing apoptosis.

**Effect of DHA on preadipocyte differentiation.** The representative images of Oil Red O staining demonstrated that DHA suppressed lipid accumulation (Fig. 3A). This was supported by quantitative data on image analysis. DHA dose-dependently decreased the percentage of lipid area and the number of lipid droplets during differentiation (\( P < 0.01 \), Fig. 3B,C). These observations were confirmed by GPDH activity (Fig. 4). The enzyme activity was 47.6, 26.9, and 23.9% of control values in cultures containing 25, 50, and 200 \( \mu \text{mol/L} \) DHA, respectively (\( P < 0.01 \)). Thus, treating 3T3-L1 cells with DHA during induction resulted in inhibition of adipocyte differentiation.

**Effect of DHA on adipocyte lipolysis.** DHA increased basal lipolysis in fully differentiated 3T3-L1 adipocytes compared with control (Fig. 5, \( P < 0.01 \)). Free glycerol release into the culture medium was increased by \(-50 \) (50 and 100 \( \mu \text{mol/L} \) DHA) and \(90\% \) (200 \( \mu \text{mol/L} \) DHA) after 4 h incubation.

However, DHA had no additional effect on epinephrine-induced lipolysis at the epinephrine dosage used (data not shown).

**Discussion**

Feeding fish oil rich in (n-3) PUFA was shown to decrease adipose tissue mass and suppress visceral fat accumulation in rats (1,2). There have been few studies on the relation between (n-3) PUFA and adiposity in humans due to the difficulty in the control of other variables. A cross-sectional study in Japan showed that BMI in women was negatively associated with dietary intake of (n-3) PUFA and DHA (22). In the study, plasma DHA levels ranged from 0.31 to 0.62 mmol/L, ~4 to 5.5% of total fatty acids. The concentration of DHA in our study was based on the physiological level of FFA unesterified circulating lipid. It was reported that plasma FFA levels were 0.5–2.3 mmol/L in rats (4) and 0.25–0.73 mmol/L in humans (23). We selected 25–200 \( \mu \text{mol/L} \) as the experimental doses in our in vitro study.

In this study, we found that DHA had antiadipogenic effects during differentiation in 3T3-L1 cells. DHA treatment during
Figure 5 Effects of DHA treatment on 3T3-L1 adipocyte lipolysis. Fully differentiated adipocytes were treated for 4 h with DHA (50, 100, and 200 μmol/L) or vehicle. Values are means ± SEM, n = 4 replicates, for glycerol released per well and are presented as percentage of control. Means without a common letter differ, P < 0.05.

differentiation suppressed lipid accumulation, as shown by dose-dependent decreases in mean droplet size and percentage of lipid area. This is consistent with Madsen et al. (24), who observed that PUFA including DHA suppressed TG accumulation during MDI-induced differentiation in 3T3-L1 cells, whereas saturated and monounsaturated fatty acids had no effect compared with control. These morphological observations were confirmed by GPDH activity, which is a marker of the late phase of differentiation. The enzyme activity was decreased markedly, by more than one-half, even with the 25 μmol/L treatment. Okuno et al. (25) demonstrated perilla oil rich in (n-3) PUFA prevents growth of visceral adipose tissue in rats by downregulating the late phase of adipocyte differentiation. Therefore, DHA may have reduced fat deposition by suppressing lipid filling in adipocytes.

Considering that differentiation to adipocytes depends on proliferation of preadipocytes and commitment to differentiation (6), we predicted that DHA would also affect preadipocyte proliferation in addition to lipid filling. Many studies have shown that PUFA have antiproliferative properties in various types of cells (10–12). Awad et al. (26) also observed that 3T3-L1 preadipocyte growth was inhibited by DHA supplementation. However, these studies cannot exclude the possibility of peroxidative damage on cell growth, because they did not include antioxidants in the medium. Indeed, Chajes et al. (13) showed that (n-3) PUFA were effective to arrest MCF-7 cell growth but addition of Vitamin E restored cell growth. We supplemented α-tocopherol in the medium to protect DHA from peroxidation to determine the effect of DHA itself. α-Tocopherol alone had no effect on 3T3-L1 cell growth and differentiation from our preliminary tests and a published study (27). No appreciable change in MTS assay results and LDH release in preconfluent cells suggests that DHA did not affect cell growth in 3T3-L1 preadipocytes. However, after initiation of differentiation, DHA treatment decreased cell viability with an increase in LDH release.

Adipocyte differentiation is accompanied by the occurrence of 2 critical events during the first few days after initiation of differentiation: mitotic clonal expansion and an irreversible commitment to differentiation (6,28). The DHA-mediated decrease in TG accretion was accompanied by growth arrest and cell death during the mitotic clonal expansion phase. It is possible that the inhibition of mitotic clonal expansion was accompanied by initiation of apoptosis or necrosis. LDH release into the cell medium has been used as a cytotoxicity index, but it does not distinguish between cell necrosis and late phase of apoptosis involved in cell death. Our single-stranded DNA ELISA results demonstrate a pronounced apoptotic effect of DHA after 24 and 48 h of treatment. To our knowledge, this is the first report to demonstrate that DHA has caused apoptosis in preadipocytes. Considering that postconfluent 3T3-L1 preadipocytes undergo several rounds of replication during the first 48 h of the differentiation, the induction of apoptosis in postconfluent differentiating cells may be a potential mechanism by which DHA attenuates adipogenesis, leading to fewer adipocytes. Therefore, we suggest that DHA inhibits lipid accumulation at least in part by inducing apoptosis in postconfluent preadipocytes.

The effect of DHA on cell growth and differentiation may be caused by altering cellular membrane phospholipid composition and intracellular metabolism. In fact, a significant incorporation of DHA and decreased content of arachidonic acid in membrane phospholipids of fat tissue was observed when feeding diets rich in (n-3) PUFA to rats (23). One possible mechanism involves the alteration of prostaglandin synthesis. It is conceivable that DHA may suppress adipocyte differentiation by downregulating prostaglandin synthesis from arachidonic acid. Prostaglandins, especially PGI2 and PGF2α, are closely related with cell proliferation and terminal differentiation of adipocytes (24). Indeed, DHA was found to suppress cell growth by reducing PGE2 formation in 3T3 fibroblasts (29). Recently, it was reported that the selective cyclooxygenase-2 inhibitor, celecoxib, had a synergistic effect on DHA-induced apoptosis in colon cancer cells (30). Therefore, changes in eicosanoid biosynthesis may be responsible for suppression of adipogenesis and/or induction of apoptosis.

Alternatively, DHA could be influencing esterification into TG. Previous studies showed that saturated fatty acids and monounsaturated fatty acids are more readily acylated into TG in adipose tissue than PUFA (31) and that during differentiation of 3T3-L1 cells, PUFA suppress de novo fatty acid synthesis, whereas saturated and monounsaturated fatty acids do not (24).

In addition to observing the antiadipogenic effect, this study also found that DHA stimulated lipolysis when added to mature adipocytes. The few animal studies that examined the effects of (n-3) PUFA on lipid mobilization support our finding in 3T3-L1 cells. They reported that lipolysis was increased in isolated adipocytes derived from dietary fish oil or (n-3) fatty acid–fed animals (4). In addition, Raclot and Groscolas demonstrated the preferential mobilization of highly unsaturated fatty acids such as DHA and EPA in vitro (32). Therefore, increased lipid mobilization from adipocytes by lipolysis could be another explanation for the reduction in body fat reported following DHA treatment in rodents.

DHA increases fatty acid oxidation and energy expenditure by activating PPARα and inducing UCP-2 expression in white fat (33,34) and DHA suppresses fatty acid synthesis by inhibiting sterol regulatory element-binding protein in the liver (35). However, the detailed action of DHA in 3T3-L1 cells is largely unknown and further studies are needed at the molecular level to elucidate cellular mechanisms involved in DHA’s effects on differentiation and lipid metabolism in adipocytes.

We have shown that DHA functions directly in at least 2 different ways to inhibit adipocyte development: inhibition of differentiation-associated mitotic clonal expansion in postconfluent preadipocytes and their subsequent lipid accumulation in adipocytes. In addition, apoptosis in postconfluent preadipocytes could partly contribute to the reduction of cellularity. In conclusion, these data demonstrate that DHA acts primarily by reducing preadipocyte differentiation, inducing apoptosis, and promoting adipocyte delipidation; therefore, it could mediate a reduction of body fat.


