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Differential responses to docosahexaenoic acid in primary and immortalized cardiac cells

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HIGHLIGHTS

- Investigated the response to DHA in H9c2 cardiac cells and neonatal cardiomyocytes.
- DHA treatment decreased cell viability in H9c2 cells.
- DHA treatment increased caspase-3 activity and IL-6 release in H9c2 cells.
- DHA induced mitochondrial damage in H9c2 cells.
- DHA treatment is detrimental to H9c2 cells but not neonatal cardiomyocytes.

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ABSTRACT

The importance of dietary polyunsaturated fatty acids (PUFAs) in the reduction of cardiovascular disease has been recognized for many years. Docosahexaenoic acid (22:6n3, DHA) is an n-3 PUFA known to affect numerous biological functions and provide cardioprotection; however, the exact molecular and cellular protective mechanism(s) remain unknown. In contrast, DHA also possesses many anti-tumorgenic properties including suppressing cell growth and inducing apoptosis. In the present study, we investigated the effect of DHA toward H9c2 cells (an immortalized cardiac cell line) and neonatal primary cardiomyocytes (NCM). Cells were treated with 0 μM, 10 μM or 100 μM DHA for upto 48 h. Cell viability and mitochondrial activity were assayed at different time points. DHA caused a significant time- and dose-dependent decrease in cell viability and mitochondrial activity in H9c2 cells but not NCM. In addition, DHA decreased levels of TGF-β1 but increased IL-6 release in H9c2 cells. Significant induction of apoptosis was observed only in H9c2 cells, which involved activation of caspase-8 and -3 activities with a marked release of cytochrome c from mitochondria. DHA-induced severe mitochondrial damage resulting in a fragmented and punctated morphology with corresponding loss of mitochondrial membrane potential within 3 h, prior to activation of caspases and cytochrome c release at 6 h in H9c2 cells. Our data indicate that DHA treatment targets mitochondria, triggering collapse of mitochondrial membrane potential, increasing cellular stress and mitochondrial fragmentation resulting in apoptosis in immortalized cardiac cells, H9c2, but not neonatal primary cardiomyocyte.

1. Introduction

Polyunsaturated fatty acids (PUFAs) have critical structural and functional roles in ensuring cellular homeostasis. While oxidation of fatty acids represents an important primary source of energy in many cell types, they can also act as important lipid mediators regulating cellular signal transduction pathways. The inability of mammals to synthesize most PUFAs endogenously stems for the lack of enzymes necessary to form double bonds (desaturation) or add carbon atoms (elongation), as such they rely on dietary sources. n-3 PUFAs, including α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are a major group of PUFAs that have a double bond at carbon position 3 (omega). Another important group of PUFAs are n-6-PUFAs, which have a double bond at carbon 6, including linoleic acid (LA,

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18:2n-6) and arachidonic acid (AA, 20:4n-6) (Harris et al., 2008; Serini et al., 2011).

The importance of dietary PUFAs in the reduction of cardiovascular disease (CVD) has been recognized for many years (Erkkila et al., 2008; Kritchevsky, 1998). Early studies of dietary fat and atherosclerosis investigating the association of serum cholesterol with coronary heart disease suggested that unsaturated fatty acids lowered serum cholesterol levels (Keys et al., 1957; Mensink and Katan, 1992; Mensink et al., 2003; Page, 1954). A typical modern Western diet contains high amounts of saturated fatty acids (SFAs) and n-6 PUFAs that can negatively influence CVD through effects on thrombosis, endothelial function, inflammation and obesity (Adkins and Kelley, 2010). While, increased consumption of n-3 PUFAs has been linked with reduced risk of CVD, myocardial infarction, cardiac arrhythmias, sudden cardiac death, atherosclerosis and hypertension (Wang et al., 2006). Several mechanisms have been proposed to explain the cardioprotective effects of n-3 PUFAs, most notably for EPA and DHA, such as improving cardiac function (Peltier et al., 2006), preventing arrhythmia (Billman et al., 1999) improving heart recovery (Mancardi et al., 2009) and reducing infract sizes (Mancardi et al., 2009; Xiao et al., 2008). However, the appropriate dosages and the mechanism(s) behind the beneficial effect of n-3 PUFA remain unclear (Anderson et al., 2012; Nair et al., 1997).

Conversely, recent studies have demonstrated that DHA can orchestrate the regulation of programmed cell death in cancer cells (Blanckaert et al., 2010; Kang et al., 2010). DHA has a profound ability to suppress growth of breast, prostate and colon tumors (Astorg, 2004; Karmali, 1989; Skender et al., 2012) and to selectively sensitize tumor cells to chemotherapeutic agents without affecting normal cells (Hajjaji and Bougnoux, 2012). Discrepancies in experimental design and interpretation of results have hindered identification of differences in response between normal and immortalized cells. While the exact mechanism(s) remain largely unknown, several pathways have been proposed including increased lipid peroxidation and activation of apoptosis (Chen and Istfan, 2000; Das, 1999; Larsson et al., 2004). Indeed, understanding of the molecular mechanisms underlying this phenomenon will unmask the therapeutic potential of DHA. Identifying differences in the cell signaling pathways between normal and tumor cells is very important particularly for the development of highly selective chemotherapeutic agents.

Given a dearth of differential effects observed with DHA and the lack of mechanistic information, the aim of the current study was to investigate the effects of increasing concentrations of DHA in immortalized cardiac cells, H9c2 cells, and primary cardiac cells, rat neonatal cardiomyocytes. Our data highlight major differences in cellular responses between the two cell models and demonstrate that DHA treatment has a selective cytotoxic effect on H9c2 cells with no effect on NCM.

2. Materials and methods

2.1. Chemicals and reagents

DHA (250 mg/ml) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Tissue culture materials were obtained from Invitrogen (Burlington, ON, Canada); Bradford protein assay solution from BioRad Laboratories (Mississauga, ON, Canada); ECL Plus chemiluminscence solution from Amersham Biosciences (Parti Québécois, QC, Canada); primary and secondary antibodies from Cell Signaling (Pickering, ON, Canada); digitonin, Trypan blue, MTT and total antioxidant capacity assay kit from Sigma–Aldrich (Oakville, ON, Canada); total 20S proteasome assay kit from Chemicon (Millipore), (Etobicoke, ON, Canada); TGF-β1 and IL-6 assay kits from Abcam (Toronto, ON, Canada).

2.2. Cell culture

H9c2 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 37 °C (5%CO_2/95%N_2). NCM were isolated form rat pups (1–3 day old) as previously described (Kovacic et al., 2003) and cultured in DMEM supplemented with 15% horse serum, 5% FBS, and 1% penicillin and streptomycin at 37 °C (5%CO_2/95%N_2). Cells were treated with 0 μM, 10 μM or 100 μM DHA for 0 h, 12 h, 24 h, 36 h or 48 h, harvested and analyzed.

2.3. Western blotting and cytochrome c release

Release of cytochrome c (Cyt c) from mitochondria was measured as previously described (Seubert et al., 2002). Briefly, H9c2 cells were washed with ice-cold PBS and then harvested and centrifuged at 1500 × *g* for 10 min. Supernatants were removed and pellets were suspended in 200 μl of lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM $NaHPO_4$, 250 mM sucrose, 5 μg/ml digitonin and 5 μl/ml protease inhibitors). Cell suspensions were incubated on ice for 5 min and then centrifuged at 14,000 × *g* for 10 min (4 °C). Supernatants were used as cytosolic fraction. Pellets (heavy membrane fraction) were re-suspended in Tris–HCl buffer (25 mM Tris–HCl, pH 8.0, and 0.1% Triton X-100). Samples (20 μg protein) were resolved in SDS-polyacrylamide gel and then transferred electrophoretically to nitrocellulose membranes, which were blocked in TBS-T buffer (0.15 M NaCl, 3 mM KCl, 25 mM Tris hydroxymethyl methylamine and 0.1% Tween-25, pH 7.4) with 5% skim milk for 2 h at room temperature. Membranes were washed three times with TBS-T buffer (allowing 15 min-interval between washes) and then incubated with anti-cytochrome c antibody (1:1000, Cell Signalling, catalog #4280) overnight at 4 °C. Membranes were washed as described above and then incubated with horseradish-peroxidase linked anti-rabbit IgG secondary antibody (1:10,000, Cell Signalling, catalog #7470) for 2 h at room temperature. Membranes were washed as described above. Chemiluminscence solution was used to detect signals. Relative band intensity (expressed in arbitrary units) was measured using Image J software (NIH, USA).

2.4. Cell viability

Cell viability was estimated based on a modified Trypan blue exclusion method as described previously (Batchu et al., 2011). Briefly, 0.4% of Trypan blue solution was added to cells for 15 min at room temperature. Then cells were centrifuged at 1000 × *g* for 5 min at room temperature. The supernatant was gently aspirated and cells were washed three times with ice-cold PBS. 1% sodium dodecyl sulfate solution was added to lysed cells. Optical density was read spectrophotometrically at 595 nm.

2.5. Enzymatic assays

Cellular activity was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) to formazan crystals as described previously (Batchu et al., 2012). MTT assay is a widely used colorimetric assay for estimation of cell survival and cellular activity. Briefly, MTT solution was added to incubated cells for 6 h at 37 °C prior to assessment. Medium was aspirated and cells were allowed to air-dry at room temperature. Dimethyl sulfoxide was added to dissolve formazan crystals (formed by oxidation of MTT). Optical density was measured spectrophotometrically at 490 nm and cellular activity ratio was represented relatively to control.

Transforming growth factor beta 1 (TGF-β1) and interleukin-6 (IL-6) were measured in the cell culture supernatants by ELISA (Abcam, catalog #ab64715; and Abcam, catalog #ab6672). 20S total proteasome activity was measured using an assay kit (Chemicon, catalog #APT280) based on the detection of 7-amino-4-methylcoumarin (AMC) fluorescence in cell lysates after cleavage of the peptide LLVY-AMC. Fluorescence was measured at 380 nm excitation and 460 nm emission. A standard curve was established with AMC.

Caspase-3 and -8 were assessed as described previously, using the specific substrates Ac-DEVD-AMC (caspase-3) and Ac-IETD-AMC (caspase-8) (Seubert et al., 2002). In brief, cells were plated at 2×10^5 cells/well (six-well plate), grown until 90% confluence, treated with DHA and harvested after 24 h of treatment. Cells were centrifuged at 500 × *g* for 2 min and the supernatants were discarded. The pellets were re-suspended (after washing with PBS) in 200 μl of lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, and 10 mM $NaPP_i$) followed by centrifugation at 15,000 × *g* for 15 min (4 °C). Caspase activity was determined in cell lysates by detection of AMC fluorescence after cleavage of the peptide. The fluorescence was monitored at wavelengths of 380 (excitation) and 460 nm (emission). The activity was calculated by using a linear standard curve found with AMC.

Total antioxidant capacity was measured in the cell lysates based on the formation of a ferryl myoglobin radical from myoglobin and hydrogen peroxide which oxidizes ABTS [2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] to $ABTS^{\bullet+}$ (soluble green chromogen) which can be detected spectrophotometrically at 405 nm (Sigma kit, catalog #CS0790-1KT). In the presence of antioxidants, the $ABTS^{\bullet+}$ is suppressed to an extent dependent on the activity of the antioxidants and the color intensity is decreased proportionally. Trolox, a water-soluble vitamin E analog, serves as a control antioxidant.