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Liposome-incorporated DHA increases neuronal survival by enhancing non-amyloidogenic APP processing

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ABSTRACT

The fluidity of neuronal membranes plays a pivotal role in brain aging and neurodegeneration. In this study, we investigated the role of the omega-3 fatty acid docosahexaenoic acid (DHA) in modulation of membrane fluidity, APP processing, and protection from cytotoxic stress. To this end, we applied unilamellar transfer liposomes, which provided protection from oxidation and effective incorporation of DHA into cell membranes. Liposomes transferring docosanoic acid (DA), the completely saturated form of DHA, to the cell cultures served as controls. In HEK-APP cells, DHA significantly increased membrane fluidity and non-amyloidogenic processing of APP, leading to enhanced secretion of sAPPα. This enhanced secretion of sAPPα was associated with substantial protection against apoptosis induced by ER Ca^{2+} store depletion. sAPPα-containing supernatants obtained from HEK-APP cells exerted similar protective effects as DHA in neuronal PC12 cells and HEK293 control cells. Correlating to further increased sAPPα levels, supernatants obtained from DHA-treated HEK-APP cells enhanced protection, whereas supernatants obtained from DHA-treated HEK293 control cells did not inhibit apoptosis, likely due to the low expression of endogenous APP and negligible sAPPα secretion in these cells. Further experiments with the small molecule inhibitors LY294002 and SP600125 indicated that sAPPα-induced cytoprotection relied on activation of the anti-apoptotic PI3K/Akt pathway and inhibition of the stress-triggered JNK signaling pathway in PC12 cells. Our data suggest that liposomal DHA is able to restore or maintain physiological membrane properties, which are required for neuroprotective sAPPα secretion and autocrine modulation of neuronal survival.

1. Introduction

The amyloid precursor protein (APP) and its metabolism play fundamental roles in the pathophysiology of familiar and sporadic Alzheimer's disease (AD), which presents the most common neurodegenerative disorder and one of the leading causes of death in the elderly. Cleavage of APP at the N-terminus by β-secretase produces sAPPβ and the membrane-associated C-terminal fragment C99. Subsequent cleavage of C99 by γ-secretase generates AICD along with varying forms of extracellular Aβ [1]. Current research suggests that oligomeric forms of Aβ have a major impact in AD [1,2]. Under physiological conditions, the majority of APP is processed by the activity of α-secretase at the cell surface within the Aβ sequence [3]. α-Secretases belong to the ADAM (a disintegrin and metalloprotease) family [4] and via their metalloprotease domains, ADAMs are implicated in ectodomain shedding of many surface molecules (e.g., growth factors) and in initiation of intracellular signaling via regulated intramembrane proteolysis (RIP). ADAMs 9, 10, and 17 have been proposed to act as α-secretases for APP [5]. Proteolytic cleavage of APP by ADAM enzymes produces a membrane-associated C-terminal fragment with a size of 83 amino acids (C83) and a 105-125 kDa soluble and secreted N-terminal APP fragment (sAPPα). Although altered APP processing and aberrant expression of APP are known to be crucially involved in the pathophysiology of AD [1,6,7], the physiological functions of APP and its cleavage products are still not well understood. However, sAPPα has been reported to have neurotrophic functions in the central nervous system [7]. Moreover, accumulating evidence suggests that APP is implicated in regulation of

Abbreviations: ADAM, a disintegrin and metalloprotease; AD, Alzheimer's disease; APP, amyloid precursor protein; ATP, adenosine-triphosphate; BSA, bovine serum albumin; CTF, C-terminal fragment; DA, docosanoic acid; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; JNK, c-Jun N-terminal kinase; LS, liposomes; MMP, mitochondrial membrane potential; MLV, multilamellar vesicles; NPD1, neuroprotectin D1; PC, phosphaditylcholine; PUFA, polyunsaturated fatty acid; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; TBS, Tris-buffered saline

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gene expression [8–10]. In AD, lowered levels of sAPPα were found in the cerebrospinal fluid of AD patients [11–13] and strategies to improve α-secretase activity may provide neuroprotection [14]. Stimulation of α-secretase activity can be achieved via several signaling cascades including phospholipase A2 and C, phosphatidylinositol 3-kinase or serin/threonin-specific kinases [4,15,16].

Polyunsaturated fatty acids (PUFAs) are essential for regulating the fluidity of cell membranes, and there are several lines of evidence that the fluidity of membranes is important for modulation of APP processing [17–19]. In the present study, we focused on DHA, which represents the most abundant PUFA in the brain [20] and is implicated in various functions including mediation of membrane–protein interactions [21], gene expression [22], neurogenesis [23–26], and learning [27–29]. DHA can also exert neuroprotective effects, e.g., against neurotoxicity induced by Aβ [30–34].

Recently, we have reported that overexpression of APP protects PC12 cells against apoptosis triggered by endoplasmic reticulum (ER) stress and genotoxic stress [10,35]. Furthermore, APP was able to inhibit stress-triggered activation of the c-Jun N-terminal kinase (JNK) pathway [10], which represents a central stress signaling pathway implicated in many paradigms of neuronal and non-neuronal cell death [36]. Previous studies have also suggested that the neuroprotective effects of DHA may be correlated to modulation of JNK signaling [31,37], but the functional relevance of APP/sAPPα has not been addressed in this context. Here, we investigated the cytoprotective properties of DHA and sAPPα in models of ER Ca^{2+} store depletion-induced cell death. Our data demonstrate that sAPPα plays an important role in mediating the protective effects of DHA. Furthermore, they suggest that autocrine, sAPPα-dependent neuroprotection involves activation of the pro-survival PI3K/Akt pathway [38] and inhibition of stress-triggered JNK activation.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all cell culture reagents were obtained from Gibco/Invitrogen. Trimethylammoniumdiphenylhexatrien (TMA-DPH), DHA, DA, 3,5-Di-tert-4-butylhydroxytoluene (BHT), and the PI3K/Akt inhibitor LY294002 were obtained from Sigma-Aldrich (Taufkirchen, Germany). The caspase substrate acetyl-DEVD-7-amino-4-methylcoumarin and Thapsigargin were purchased from Axxora (Lörrach, Germany). The JNK inhibitor SP600125 was obtained from Calbiochem. Lipoid S100® was a kind gift from Lipoid (Ludwigshafen, Germany).

2.2. Antibodies

C-terminal fragments (CTF) and secreted sAPPα were detected using monoclonal mouse IgG 6E10 (Signet Laboratories, Cat.9320-02), which recognizes residues 1–17 of Aβ. Cellular levels of APP were detected using a polyclonal antibody obtained from Sigma-Aldrich (A8717). Mouse anti-GAPDH was obtained from Chemicon (Hofheim, Germany, Cat. MAB374), and rabbit anti-Phospho-JNK was obtained from Cell Signaling (Cat. 4668). Anti-mouse HRP-conjugated secondary antibody was purchased from Calbiochem (Bad-Soden, Germany, Cat. 401253). Also IRDye800CM-conjugated goat anti-rabbit antibody and IRDye800CM- or IRDye680CM-conjugated goat anti-mouse antibody were obtained from LI-COR.

2.3. Preparation of liposomes

Liposomes were prepared as previously described with slight modifications [39]. Briefly, phosphatidylcholine (PC) from soy (Lipoid S100®) was dissolved in dichloromethane (37.7 mg/ml). DHA and DA were dissolved in ethanol (containing 0.05% BHT) to a final concentration of 5 mmol/l, respectively. Both solutions were mixed in a flask and dried to a thin film by rotation under reduced pressure at 30 °C. Afterwards, the lipid film was dried at low pressure in a desiccator at 4 °C for 12 h. An aliquot of distilled water (5 ml) and glass beads (5 g) were added to the flask under a stream of N_2. The flask was swiveled for 1 min and then sonicated for 1 min in an ultrasonic bath at room temperature. This procedure was repeated three times to produce multilamellar vesicles (MLV). The mixture was transferred in polycarbonate tubes, and MLV were pulsative sonicated for 45 min at 4 °C in the dark using a Branson-Sonifier (Typ Cell Disruptor B15, Firma Branson Ultrasonics Corp) under a stream of N_2 to prepare small unilamellar vesicles (SUV). The liposome suspension was centrifuged at 8000×*g* for 10 min to remove debris. The supernatant was removed and centrifuged again at 15,000×*g* for 5 min. A volume of 4 ml containing SUVs was removed from the top of the tube and sterile filtered through a 0.22-μm millipore filter. Analogically, control liposomes without the addition of fatty acids were prepared. The size distribution of liposomes was determined using photon correlation spectroscopy (Zetasizer 3000 HSa, Malvern Instruments, Malvern, Worcestershire, UK). Incorporation of DHA was checked using gas-chromatography and flame ionization detection.

2.4. Cell culture

HEK293 cells, stably transfected with human APP695, were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C and 5% CO_2. The HEK-medium was supplemented with 10% FCS and penicillin/streptomycin. Geniticin (G418) was added at 3 μg/ml as a selective antibiotic. Untransfected HEK293 cells were cultured in the same medium without G418. PC12 cells were cultured with DMEM with high glucose supplemented with 10% FCS, 5% horse serum, and penicillin/streptomycin. HEK cells were incubated for 5 days with liposomes as follows: On day 0, cells were plated and incubated with control liposomes, liposomes containing DHA (final concentration 20 μM) or liposomes containing DA (final concentration 20 μM). On days 2 and 4, media were changed and new liposome solutions were added. On day 5, media were collected and cells were harvested for further examinations.

2.5. Cytotoxicity

Cytotoxicity in the current study was determined using the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously reported [40]. No cytotoxicity was observed for the concentrations of inhibitors and substances used in the current study (data not shown).

2.6. Determination of lipid peroxidation

Malondialdehyde (MDA) levels were determined using a microplate assay for lipid peroxidation (Lipid Peroxidation Microplate Assay Kit FR22; Oxford Biomedical Research, Oxford MI, USA) according to the manufacturer's instructions. This assay is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA at 45 °C. Briefly, cells were cultured in serum containing medium as described above. Prior to homogenization, cells were washed several times to remove serum components. Cells were lysed by sonication. To prevent sample oxidation during preparation, lysis was done in the presence of 10 μl 0.5 M BHT per 1 ml of cell homogenate. After lysis, homogenate was centrifuged at 3000×*g* and 4 °C for 10 min to remove debris. An aliquot of the sample was saved for protein determination. The sample was immediately frozen at −70 °C prior to testing.