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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.

2.7. Determination of protein oxidation

Proteincarbonyl levels were determined using a microplate assay for protein oxidation (OxiSelect Protein Carbonyl Spectrophotometric assay STA-315; Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, protein carbonyls in cell homogenates were derivatized with dinitrophenylhydrazine (DNPH) first. Proteins were then TCA precipitated, and free DNPH was removed by washing the protein pellet. After dissolving the protein pellet in GuHCl, the absorbance of protein-hydrozones was measured at 375 nm, and the protein carbonyls were calculated. Oxidized bovine serum albumine (BSA) was used as positive control.

2.8. Fatty acid analysis

Fatty acids were analyzed as previously described [41]. Briefly, after addition of internal standards (C13:0, C17:0, and C19:0), total lipids were extracted from cell homogenate using methanol/chloroform (1:2 v/v) and evaporated to dryness. The lipid residue was reconstituted and esterified using boron trifluoride. Fatty acid methyl esters were than subjected to gas-chromatography (Column: BPX70 30 m capillary column, 0.25 mm id, 0.25-μm film thickness) coupled with flame ionization detection (6890N Agilent GC equipped with a 7683B Auto injector/Auto sampler), and relative measures were calculated.

2.9. Determination of membrane fluidity in living cells

The membrane fluidity of living cells was determined as earlier described [42]. The fluorescence polarization probe TMA-DPH incorporates very rapidly into plasma membranes of cells and is specifically localized on the cell surface making its use particularly appropriate for determining plasma membrane fluidity in living cells [43]. Cells were washed twice with warm HBSS and supplemented with 2 μM TMA-DPH. 700,000 cells per vial were incubated at 37 °C for 20 min. The membrane fluorescence polarization was determined using a SLM Aminco Bowman Series 2 luminescence spectrometer. The cuvette temperature was 37 °C. The steady-state fluorescence polarization (P_s) was expressed as the anisotropy (r_s) of the probe, using the following equation: $r_S = 2P_S/3 - P_S$. The r_S is inversely correlated to the membrane fluidity, particularly to the acyl-chain flexibility of phospholipids.

2.10. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) in living cells was determined using Rhodamin123 as fluorescence probe with slight modifications as previously described [44]. Briefly, cells were incubated in the dark for 15 min with 0.4 μM R123 on a horizontal shaker. Cells were twice both centrifuged (1500 rpm, 5 min) and washed with HBSS buffer (supplemented with Mg^{2+}, Ca^{2+} and HEPES, pH 7.4, 37 °C). After supplementing cells with new HBSS, MMP was assessed by flow cytometry (FACSCalibur) using Cell Quest software (BD Biosciences).

2.11. Determination of cellular ATP levels

Cellular levels of adenosine-triphosphate (ATP) were determined using a chemiluminescence-based kit (Vialight Plus, Lonza, Rockland, USA) according to manufacturer's instruction as previously described [44].

2.12. Western blot analysis

Total protein levels were determined by the Lowry method. The samples were generated by adding an appropriate volume of lysis buffer (68.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, and protease and phosphatase inhibitor) to the cells. Then the cells were immediately scraped off the plates and transferred to a microcentrifuge tube and sonicated. 50 μg protein samples were loaded onto 8% or 12% SDS-polyacrylamide gels. For the detection of released sAPPα, the conditioned medium was collected and the values were normalized to the cell lysate protein concentration. Proteins were separated at 160 V and then blotted to nitrocellulose membranes (Protean BA83, 2 μm, Whatman, Dassel, Germany) in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v) and 0.01% SDS) at 70 mA for 1 h. The blots were blocked in blocking buffer (5% non-fat milk powder, 15 mM Tris/HCl pH 7.5, 200 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h. For detection of the activated JNK1 and JNK2, we diluted the rabbit polyclonal anti P-JNK antibody 1:1000 in Tris-buffered saline (TBS) containing 5% BSA and 0.1% Tween 20 and incubated the blots overnight at 4 °C. APP and sAPPα levels were detected by diluting the appropriate antibody 1:500 in TBS-containing 3% non-fat milk powder and 0.1% Tween 20. Even these blots were incubated overnight at 4 °C. Primary antibodies were detected by subsequent incubation with IRDye800CW anti-rabbit or IRDye680CW anti-mouse or anti-rabbit in blocking solution for 1 h at room temperature. The Odyssey Infrared Imaging System (LI-COR) was used for visualizations and quantifications.

2.13. ELISA detection of sAPPα

For the quantitative analysis of sAPPα, five million HEK-APP and HEK293 control cells were plated in 10-cm dishes. Conditioned, serum-free media were collected after 24 h and supernatants were centrifuged at 200×*g* for 4 min to eliminate cell debris. Soluble sAPPα was quantified using the IBL human sAPPα colorimetric sandwich ELISA Kit (IBL Immuno-Biological Laboratories, Hamburg, Germany) according to the supplier's instructions.

2.14. Caspase-3-like protease activity (DEVDase assay)

For measurement of caspase-3-like protease activity, cells were lysed in 200 μl lysis buffer [10 mM HEPES, pH7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5% 3-(3-cholamidopropyldimethylammonio)-1-propane sulfonate (CHAPS)]. 50 μl of this lysate was added to 150 μl reaction buffer (25 mM HEPES, pH7.5, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 3 mM DTT) and 10 μM of the fluorogenic substrate Ac-DEVD-AMC. This substrate is cleaved by caspase 3, and also by the caspases 6, 7, 8, and 10. Accumulation of Acetyl-DEVD-7-amido-4-methylcoumarin (AMC) fluorescence was monitored over 2 h using an Htsoft Tecan Genios multiplate reader (excitation 360 nm, emission 465 nm). Fluorescence of blanks containing only lysis buffer was subtracted from the values. Protein content was determined using the Roti®quant (Karl-Roth GmbH, Karlsruhe, Germany). Caspase activity is expressed as change in fluorescent units per microgram protein and per hour.

2.15. Statistics

All experiments were done at least in three independent experiments, and all assays were done in triplicates or quadruplicates. Statistical analyses were performed using one-way ANOVA followed by a Tukey comparison test. All data were expressed as the means ± SEM. The correlations were calculated using SPSS (IBM).

3. Results

3.1. DHA enhances membrane fluidity and sAPPα secretion in HEK-APP cells

In our experimental approach, cells were incubated with unilamellar transfer liposomes containing DHA to provide effective

incorporation of the fatty acid into cell membranes [45]. Levels of malondialdehyde (Fig. 1A) and of proteincarbonyls (Fig. 1B) were unchanged, confirming that the polyunsaturated fatty acids in our liposomal DHA preparations were protected from oxidation [46,47]. The cells were also incubated with pure liposomes (LS) or liposomes containing DA, which served as controls. As to be expected, incubation of HEK-APP cells with LS or DA (20 μM) for 5 days had no effect on cellular DHA levels (Fig. 1C). In contrast, incubation of cells with DHA (20 μM) resulted in potently elevated DHA levels (+420%) (Fig. 1C). Order parameters of biological membranes are decreased by the incorporation of DHA [48]. Accordingly, enhanced cellular DHA levels were accompanied by significantly enhanced membrane fluidity in living cells as determined by decreased TMA-DPH anisotropy values (Fig. 1D).

Levels of released sAPPα in supernatants of untransfected HEK293 control cells and APP overexpressing HEK293 cells were analyzed by Western blot and ELISA. The cells were seeded at equal densities and maintained in culture for 24 h. HEK-APP cells secreted significant levels of sAPPα (Fig. 1E), with the average concentration reaching 70 ng/ml after 24 h (Fig. 1E). Western blot analysis revealed that secretion of sAPPα and expression of intracellular APP was conspicuously lower in HEK293 control cells (Fig. 1F). Incubation of HEK-APP cells with DHA (20 μM) for 9 days further elevated the levels of sAPPα in the cell culture media (Fig. 1F). Concurrently, the β-secretase-related 99-residue membrane-associated C-terminal fragment C99 was clearly reduced (Fig. 1F), indicating a shift towards α-secretase processing of APP.

3.2. DHA protects HEK-APP cells from ER Ca^{2+} store depletion

In light of the fact that disturbances of Ca^{2+} homeostasis play a pivotal role in AD [49], we were interested whether DHA could protect cells from Ca^{2+} store depletion and cell death. Inhibition of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) by Thapsigargin (1 μM) significantly reduced the mitochondrial membrane potential (MMP) and ATP levels of HEK-APP cells, which had been preincubated with pure LS or LS containing DA (20 μM) for 5 days. (Fig. 2A, B). Cells incubated with LS containing DHA (20 μM) were protected from Thapsigargin-

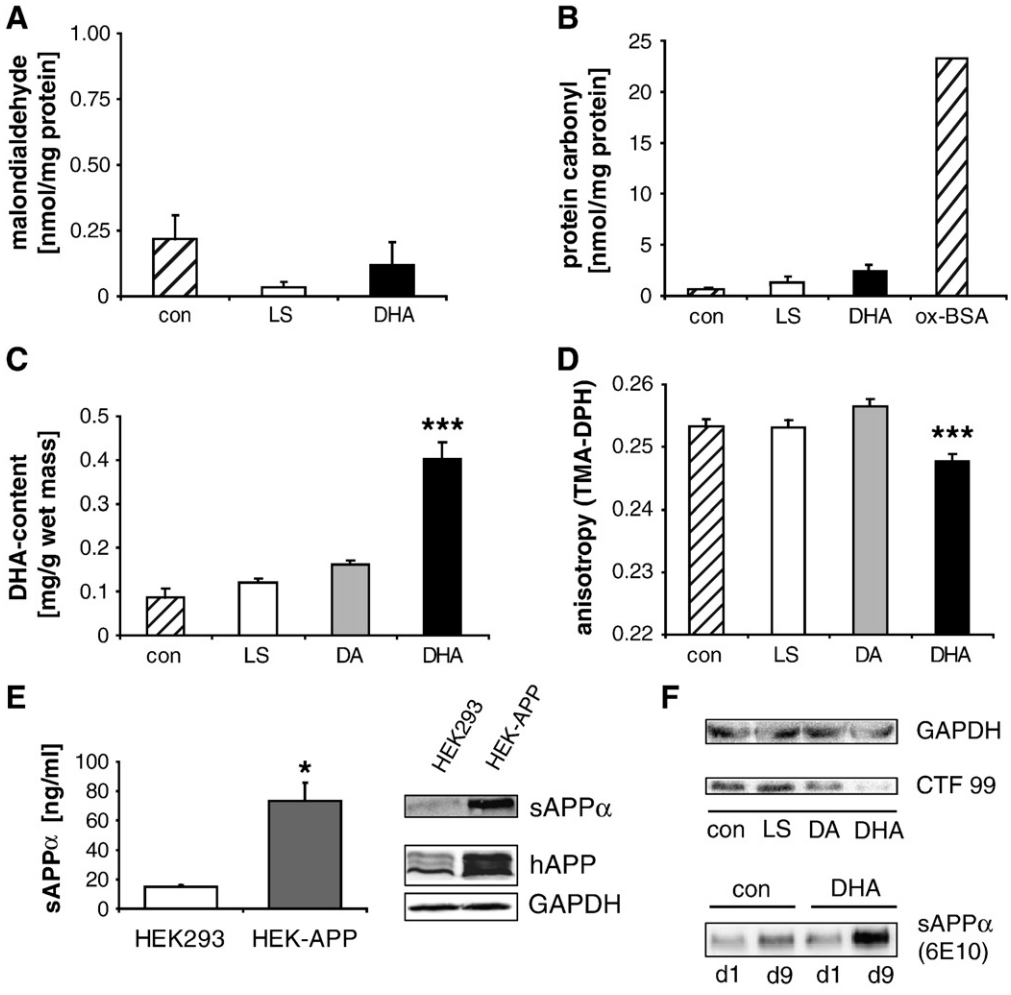

Fig. 1. DHA enhances membrane fluidity and anti-amyloidogenic processing of the amyloid precursor protein in HEK-APP cells. HEK-APP cells were incubated for 5 days with liposomes (LS), liposomes containing 20 μM DA or 20 μM DHA or were left untreated (con). ($n = 4$–6) (means ± SEM; ***$p < 0.001$). (A) Levels of malondialdehyde (nmol/mg protein) were assessed spectrophotometrically after reaction with a chromogenic reagent at 586 nm. (B) Protein carbonyl levels (nmol/mg protein) were determined after derivatization with dinitrophenylhydrazine. The amount of protein-hydrozone was measured spectrophotometrically at 375 nm. (C) Levels of DHA [mg/g wet weight] were determined using gas chromatography with flame ionization detection ($n = 4$, means ± SEM; ***$p < 0.001$). (D) Changes in membrane fluidity, which inversely correlates to the anisotropy of the fluorescence probe TMA-DPH, were determined using fluorescence polarization spectroscopy in living cells. (E) HEK-APP cells expressing human APP695 release significant amounts of sAPPα in the supernatant compared to untransfected HEK293 control cells as measured by ELISA analysis ($n = 3$) (means ± SEM; *$p < 0.05$) and by Western blot analysis in cell lysates and supernatants. The antibody directed against APP recognizes all three isoforms in the cell lysates (immature ~110 kDa, sAPP ~120 kDa, mature ~130 kDa) and sAPPα in the cell culture supernatant. GAPDH served as a loading control. (F) Levels of sAPPα were measured in cell culture supernatants and levels of C-terminal fragments of APP (CTF99) in whole cell lysates using Western blot analysis after 5 days of incubation with empty liposomes or liposomes containing DHA or DA. GAPDH served as a loading control.

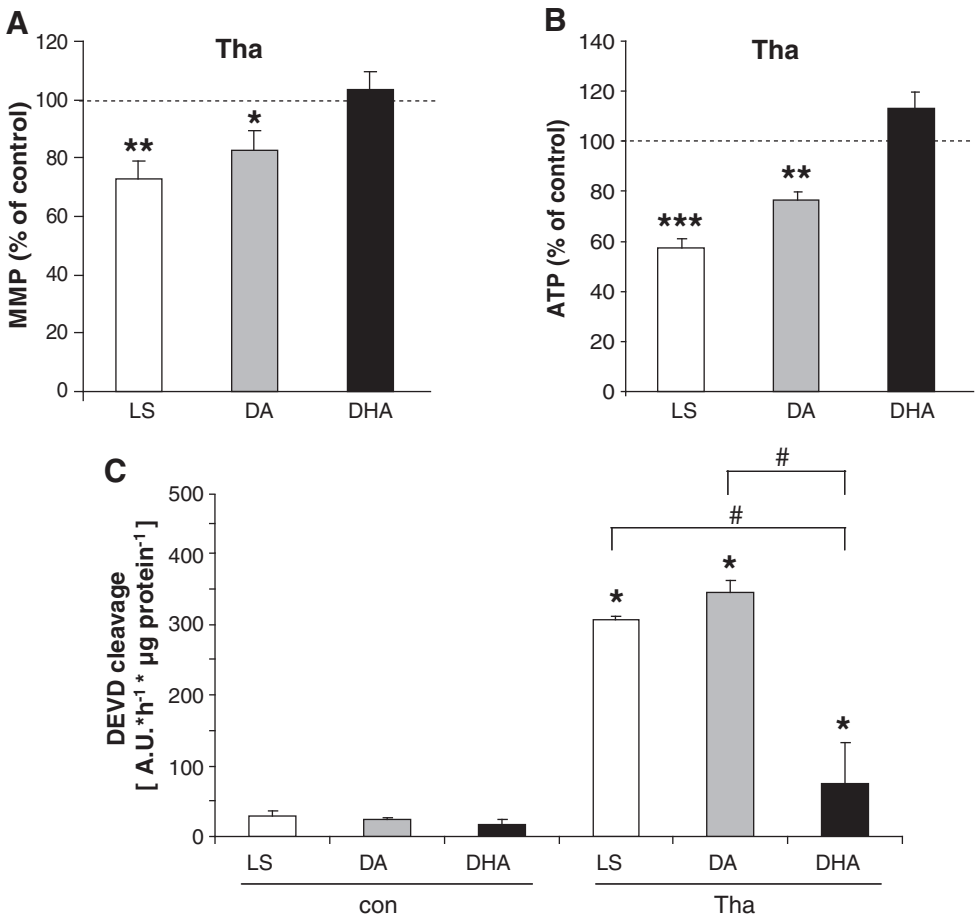

Fig. 2. DHA protects cells from ER Ca^{2+} store depletion. HEK-APP cells were incubated with liposomes (LS), liposomes containing 20 μM DA or 20 μM DHA for 5 days. Twenty-four hours before the end of treatment, cells were additionally treated with 1 μM Thapsigargin (Tha). (A) Mitochondrial membrane potentials (MMP) were determined using the fluorescence probe Rhodamin123 and FACS analysis. Values were normalized to untreated HEK-APP cell cultures ($n=6$; means ± SEM; $^{*}p<0.05$; $^{**}p<0.01$). (B) ATP levels were determined using a chemiluminescence-based assay kit. Values were normalized to untreated HEK-APP cell cultures ($n=6$; means ± SEM; $^{**}p<0.01$; $^{***}p<0.001$). (C) Caspase-3 activity was determined by DEVD cleavage assay ($n=4$) after 5 days of incubation with liposomes. Thapsigargin was added for the final 16 h of incubation at a concentration of 1 μM (AU, arbitrary fluorescence units) (means ± SEM; $^{*}p<0.05$; $^{\#}p<0.05$).

induced MMP reduction and ATP depletion (Fig. 2A, B). Thapsigargin treatment (1 μM) of HEK-APP cells incubated with LS or DA was associated with significantly enhanced effector caspase activity, indicating induction of apoptotic cell death (Fig. 2C). In analogy to its effects on stabilizing ATP levels and MMP, cells incubated with DHA (20 μM) were also protected from Thapsigargin-induced effector caspase activation (Fig. 2C).

3.3. sAPPα plays an essential role for the protective effects of DHA

Based on the observation that increased secretion of sAPPα in HEK-APP cells was associated with enhanced protection after DHA treatment (Fig. 2), we speculated that the protective effects of DHA indeed required sAPPα. To address this question, HEK293 cells were incubated with conditioned supernatants of HEK293 cells or HEK-APP cells that had been treated for 5 days either with LS alone or with LS containing DHA (20 μM), respectively. Again, HEK293 cells incubated with sAPPα-containing media obtained from untreated HEK-APP cells were significantly less vulnerable against Ca^{2+} store depletion in comparison to cells incubated with media from HEK293 cells (Fig. 3A). This protective effect against Thapsigargin-triggered stress was further enhanced when supernatants of DHA-treated HEK-APP cells were applied to the cultures (Fig. 3A). In contrast, supernatants obtained from DHA-treated HEK293 control cells did not elicit any neuroprotective effects in HEK293 cells, indicating that DHA did not stimulate secretion of sAPPα or other neuroprotectants in sufficient amounts to be detectable in our assays into the culture medium (Fig. 3A). Of note, the protective effects obtained with supernatants of DHA-treated HEK-APP cells were very similar to those observed when we directly treated HEK-APP cells with DHA (Fig. 2C). Collectively, these data suggest that sAPPα indeed plays an essential role in the protective effects of DHA.

3.4. Protective mechanisms of sAPPα involve modulation of the PI3K/Akt and JNK signaling pathways

To confirm the observed cytoprotective effects of sAPPα-containing supernatants in a neuronal cell model, we applied PC12 cells [10,35]. Cells were preincubated with conditioned culture supernatants collected from untreated HEK293 control cells and HEK-APP cells (Fig. 1C and D) after which they were challenged with Thapsigargin (1 μM) for 16 h. Activation of effector caspases was determined by cleavage of a fluorogenic substrate using the DEVDase assay [50]. PC12 cells incubated with conditioned media from HEK-APP cells were significantly less vulnerable against ER Ca^{2+} store depletion compared to cultures incubated with conditioned media from HEK293 cells (Fig. 3B, C). Our previous studies had suggested an important role of APP/sAPPα in the control of JNK/c-Jun signaling in response to cytotoxic stress triggered by UV irradiation [10]. To further investigate the molecular mechanisms of sAPPα-mediated neuroprotection, we analyzed its effects on the JNK stress signaling pathway and on the PI3K/Akt survival pathway in the experimental

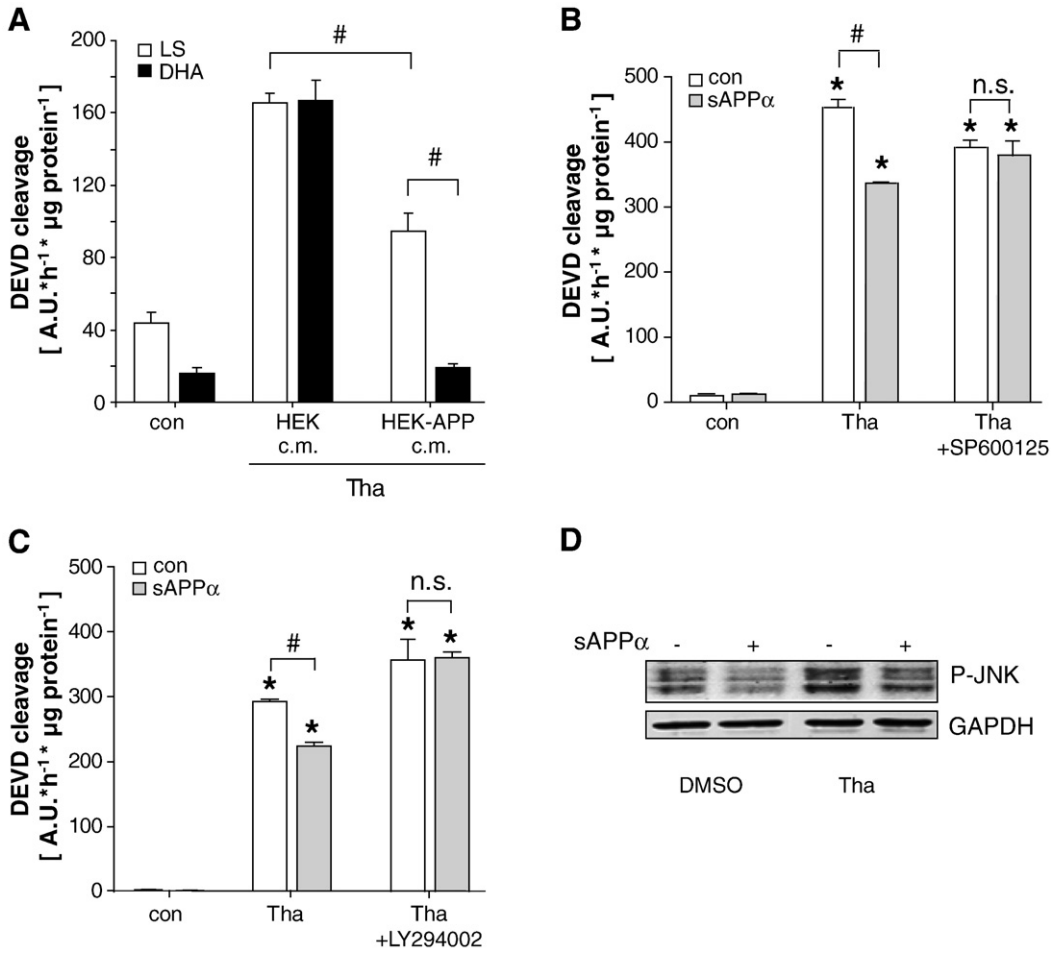

Fig. 3. Protective mechanisms of DHA are mediated by sAPPα. (A) HEK293 cells and HEK-APP cells were incubated with empty liposomes (white bars) or liposomes containing 20 μM DHA (black bars) for 5 days. HEK293 cells were incubated with the supernatants of these cultures in parallel to the treatment with Thapsigargin (1 μM) for 48 h. HEK293 c.m. indicates treatment with conditioned media derived from HEK293 control cells treated with either LS or DHA, whereas con HEK-APP c.m. indicates treatment with conditioned media derived from HEK-APP cells treated with LS or DHA. Caspase-3 activity was determined by DEVD cleavage assay ($n=4$) (means ± SEM; $^{\#}p<0.05$). (B and C) Protective effects of sAPPα involve the PI3K/Akt pathway and inhibition of the JNK pathway as analyzed with the small-molecule inhibitors LY294002 (10 μM) and SP600125 (200 nM). Cultures of PC12 cells were preincubated with conditioned media of HEK con cells (con) or HEK-APP cells (sAPPα) for 24 h. After further preincubation with or without LY294002 or SP600125 (2 h), cells were treated with Thapsigargin (1 μM) for 16 h. Caspase-3 activity was determined by the DEVD cleavage assay ($n=4$) (means ± SEM, $^{*}p<0.05$; $^{\#}p<0.05$; n.s. = not significant). (D) Thapsigargin-dependent phosphorylation of JNK is decreased after treatment of PC12 cells with sAPPα-containing supernatant as assayed by Western blot analysis. GAPDH served as a loading control. PC12 cells were treated for 6 h with 1 μM Thapsigargin.

paradigm used in this study, i.e., Ca^{2+} store depletion. Therefore, PC12 cells preincubated with conditioned supernatants of HEK293 control (con) and HEK-APP (sAPPα) cells were also treated with Thapsigargin in the presence or absence of the PI3K/Akt pathway inhibitor LY294002. Interestingly, LY294002 completely abolished the protective effects of sAPPα on apoptosis induced by Ca^{2+} store depletion (Fig. 3C). Conversely, inhibition of the JNK signaling cascade with the synthetic inhibitor SP600125 in PC12 cells preincubated with conditioned supernatants of HEK293 control cells was able to mimic the protective effects of sAPPα (Fig. 3B). Accompanying Western blot analysis demonstrated that both basal and Thapsigargin-induced JNK phosphorylation was reduced in PC12 cells incubated with sAPPα-containing media (Fig. 3D). Taken together, these data suggest that the protective effects of sAPPα are associated with activation of the PI3K signaling pathway and inhibition of the stress-activated JNK signaling pathway.

4. Discussion

Changes in the fluidity of neuronal membranes are associated with brain aging and play a central role in Alzheimer's disease [19,51,52]. The essential omega-3 fatty acids DHA and arachidonic acid are the major PUFAs in the brain [53]. PUFAs represent major components of biological membranes, and it was suggested that alterations in the architecture and function of cell membranes might contribute to the beneficial impact of *n*–3 PUFAs on cognitive functions [54–56]. There are several lines of evidence that dietary DHA can lower cognitive decline, Aβ formation, and synaptic degeneration in transgenic AD mouse models [31,32,57–60]. There is also abundant evidence that the metabolism of APP can be greatly affected by aging-associated changes in the membrane fluidity of cells [17,18,61].

Here, we studied the effects of DHA on APP processing and cell survival in APP-overexpressing HEK293 cells and neuronal PC12 cells. The fluidizing effects of DHA on membranes were previously described in cultured retinoblastoma cells [62] and in synaptosomal plasma membranes isolated from brains of Aβ-infused rats ex vivo [63]. We and others recently reported that cleavage of APP strongly depends on membrane properties and that enhanced membrane fluidity favors the non-amyloidogenic pathway of APP processing [18,19]. Accordingly, Sahlin et al. demonstrated that DHA stimulates the non-amyloidogenic APP processing in cellular models of AD [33]. In line with these findings, we demonstrate herein that liposomal

DHA is capable to increase the fluidity of cell membranes, thereby enhancing generation of sAPPα, which is known to exert autocrine and paracrine effects in neuronal and non-neuronal cells.

In previous studies, we reported that in the brain of guinea pigs treated with plant-derived omega-3 fatty acids, the levels of oleic, linoleic, arachidonic, and DHA were significantly enhanced and that dissociated brain cells isolated from these animals were less vulnerable against nitrosative stress as indicated by stabilized mitochondrial function and enhanced levels of ATP [41]. Here, we report that DHA protects HEK-APP cells against Thapsigargin-induced mitochondrial dysfunction, ATP depletion, and effector caspase activation, three hallmarks of apoptotic cell death. To address the question whether sAPPα secreted into the culture medium of DHA-treated cells was responsible for the observed protection, we compared the effects of supernatants obtained from DHA-treated HEK-APP cells and DHA-treated HEK293 control cells, respectively. In contrast to HEK-APP cells, HEK293 control cells displayed low expression of endogenous APP and negligible sAPPα secretion, and their supernatants did not provide any cytoprotection. We also demonstrated that stimulation of sAPPα secretion by DHA further enhanced the protective capacity of supernatants obtained from HEK-APP cells. From these observations, we collectively conclude that enhanced sAPPα generation significantly contributes to the cytoprotection mediated by DHA.

Since the JNK signaling pathway is an important mediator of stress-triggered neuronal cell death, which is activated in response to many pathophysiological stress stimuli implicated in neurodegeneration [10,64], we also analyzed whether sAPPα-mediated protection may be associated with alterations in the extent of JNK activation. Indeed, stress-triggered JNK phosphorylation was partly inhibited by sAPPα. As demonstrated here and elsewhere [10], sAPPα had similar protective effects after UV irradiation, suggesting that sAPPα can antagonize distinct upstream stress stimuli linked to activation of the common JNK stress signaling cascade.

There is a potential crosstalk between the pro-survival PI3K/Akt pathway and the JNK pathway, as Akt can inhibit JNK signaling by phosphorylation of the JNK upstream kinase MLK3 [65]. It was also suggested that wild-type APP, but not mutant APP, can activate the PI3K/Akt pathway [66]. Of note, the PI3K/Akt pathway inhibitor LY296002 completely abolished the ability of sAPPα to protect PC12 cells from Thapsigargin-induced cell death, suggesting that constitutive activation of the PI3K/Akt pathway might be of central relevance for the observed neuroprotective effects of sAPPα and may promote survival under various stress conditions. Interestingly, sAPPα was shown to enhance the biogenesis of the neuroprotective, DHA-derived lipid messenger neuroprotectin D1 (NPD1) [67], which was previously proposed to mediate its neuroprotective effects via the PI3K/Akt pathway [68].

Collectively, our data suggest that omega-3 fatty acids may restore or maintain physiological membrane properties, which are required for neuroprotective sAPPα secretion. Enhancing α-secretase processing of APP via physiological modulators of membrane fluidity appears to be a feasible approach for the early prevention of AD [69–71].

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