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The unfolded protein response in the therapeutic effect of hydroxy-DHA against Alzheimer's disease

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Abstract The unfolded protein response (UPR) and autophagy are two cellular processes involved in the clearing of intracellular misfolded proteins. Both pathways are targets for molecules that may serve as treatments for several diseases, including neurodegenerative disorders like Alzheimer's disease (AD). In the present work, we show that 2-hydroxy-DHA (HDHA), a docosahexaenoic acid (DHA) derivate that restores cognitive function in a transgenic mouse model of AD, modulates UPR and autophagy in differentiated neuron-like SH-SY5Y cells. Mild therapeutic HDHA exposure induced UPR activation, characterized by the up-regulation of the molecular chaperone Bip as well as PERK-mediated stimulation of eIF2α phosphorylation. Key proteins involved in initiating autophagy, such as beclin-1, and several Atg proteins involved in autophagosome maturation (Atg3, Atg5, Atg12 and Atg7), were also up-regulated on exposure to HDHA. Moreover, when HDHA-mediated autophagy was studied after amyloid-β peptide (Aβ) stimulation to mimic the neurotoxic environment of AD, it was associated with increased cell survival, suggesting that HDHA driven modulation of this process at least in part mediates the neuroprotective effects of this new anti-neurodegenerative drug. The present results in part explain the pharmacological effects of HDHA inducing full recovery of the cognitive scores in murine models of AD.

Keywords Endoplasmic reticulum stress · Unfolded protein response · Autophagy · DHA · Hydroxylated fatty acids · Alzheimer's disease

Abbreviations

Atg	Autophagy-related genes
AV	Autophagic vesicle
DHA	Docosahexenoic acid
eiF2α or eIF2α	Eukaryotic initiation factor 2 alpha
HDHA	2-Hydroxy-docosahexaenoic acid
IRE1	Inositol-requiring protein 1
UPR	Unfolded protein response
PDI	Protein disulfide isomerase
PERK	Protein kinase RNA-like ER kinase

Introduction

Over activation of the unfolded protein response (UPR) and of endoplasmic reticulum (ER) stress has been associated with a number of diseases, including neurodegenerative disorders [1]. The ER stress response protects cells from different alterations, including the excess accumulation of misfolded proteins [2]. However, when the intensity or duration of damage cannot be restored, ER stress can also

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lead to cell death by apoptosis [3]. In this context, situations that induce ER stress also activate autophagy, which induces cell death or survival [4–7].

Alzheimer's disease (AD) and other neurodegenerative diseases are characterized by the accumulation of misfolded proteins in the brain [1, 8–10]. In the case of AD, Aβ aggregates in the brain of patients as a result of sequential and anomalous β- and γ-secretase cleavage of the amyloid precursor protein (APP). This amyloidogenic processing of APP is stimulated under pathological conditions, leading to the accumulation of Aβ as fibrils that form amyloid plaques, and as soluble neurotoxic oligomers that accumulate extra- and intracellularly [11, 12]. Amyloid-β activates the three arms of UPR signaling, PERK (protein kinase RNA-like ER kinase)-eiF2α, IRE1 (inositol-requiring protein 1)-XBP1 splicing and ATF-6, which have been suggested to prevent Aβ-induced neurotoxicity [13, 14]. In this context, UPR is activated in AD brains [15–17], supporting the link between AD and ER stress. However, although UPR induction may enhance the ability of neurons to survive under these pathological conditions, if ER stress persists chronically, sustained high levels of phosphorylated eiF2α are generated that increase BACE1 levels (the main β-secretase in the mammalian brain) and amyloidogenic APP processing [18]. By contrast, some familial AD mutations and aging (the main risk factor to suffer AD) may impair the ER stress response, consequently exacerbating pathological neurodegeneration [19–21]. Together, this justifies the interest in targeting ER stress to prevent or treat AD [22].

Amyloidogenic processing of APP is thought to take place in the endo-lysosomal system, where Aβ is found in autophagic vesicles (AVs) and lysosomes [23, 24]. In fact, APP, BACE1 and the γ-secretase complex have all been located in late endosomes, AVs and lysosomes [24–27]. However, impaired turnover of AVs due to reduced vesicle fusion, impaired axonal vesicle transport or decreased lysosomal activity could lead to autophagic stress in a disease state, as seen through the accumulation of autophagic components and Aβ in dystrophic neurites and synapses [28–30]. However, the molecular role of autophagy in AD still appears to be complex and although it remains largely unclear, it is believed that the activation of autophagy would avoid the intracellular accumulation of Aβ and its precursors, dampening the impact of the AD pathology [31, 32]. This would raise the possibility of using proteins that regulate autophagy, like beclin-1, as drug targets for the treatment of AD [33–35].

We previously demonstrated that the synthetic lipid, 2-hydroxyoleic acid, induces sphingolipid metabolism alterations, ER stress/UPR and autophagy in human brain cancer (glioma) cells [36–39]. In the present work, we extended the use of hydroxylated lipids as activators of ER stress and autophagy to the treatment of AD. We previously demonstrated that HDHA restores the cognitive behavior and induces neuronal cell proliferation in a mouse model of AD based on Aβ accumulation (5xFAD mice) [40]. HDHA modulates the brain lipid membrane composition, enriching membranes in long polyunsaturated fatty acids (PUFAs) and phosphatidylethanolamine (PE) while reducing the raft-associated lipid sphingomyelin [41]. In addition, HDHA reduces the total amyloid load and tau phosphorylation in transgenic mice and in cellular models of AD [41]. Although the molecular mechanisms linking all these events are not fully understood, we provide evidence here that UPR and autophagy are involved in the mechanism of action of HDHA against neurodegeneration.

Materials and methods

Cell culture and differentiation to a neuron-like phenotype

Human neuroblastoma SH-SY5Y cells were maintained in DMEM:Hams F12 medium (1:1, Invitrogen) supplemented with 10 % FBS (Sigma), 10 units/mL penicillin/streptomycin (PAA), 1 % non-essential amino acids (Sigma) and 2 mM L-glutamine (Sigma). These cells were differentiated to a neuron-like phenotype as described elsewhere [42]. Briefly, cells were plated on poly-L-lysine pre-coated dishes and 24 h later, the medium was replaced with fresh medium supplemented with 10 μM retinoic acid (Sigma). The cells were then incubated in the dark for 5 days and the medium was replaced with serum-free medium supplemented with 50 ng/mL of human brain-derived neurotrophic factor (hBDNF, Alomone Labs, Tel Aviv, Israel). Finally, the cells were incubated for 6 days to complete their differentiation.

Differentiation was evident through the morphological changes in the cells, such as the predominant neurite projections and branches as opposed to the typical epithelial morphology of exponentially growing undifferentiated cells. Moreover, a significant loss of nestin was evident in differentiated cells by immunobloting, a typical marker of undifferentiated SH-SY5Y cells. In order to determine if differentiated SH-SY5Y had arrested their cell cycle, the cells were stained with ethidium bromide (Sigma) and the cellular DNA content was determined by single-cell fluorescence flow cytometry, as described elsewhere [39]. Differentiated SH-SY5Y cells were clearly arrested in phase G_0/G_1 of the cell cycle, as further confirmed by monitoring key proteins involved in cell cycle progression in immunoblots: cyclin-dependent kinases (Cdk4 and 6), dihydrofolate reductase (DHFR) and cyclin D3. All these proteins were markedly and significantly downregulated in differentiated SH-SY5Y cells (see online resource 1).

HDHA and DHA treatments

The HDHA we designed previously (WO2010106211 A1) was produced and provided as a sodium salt by Lipopharma Therapeutics S.L. (Palma de Mallorca, Spain), while DHA (ethyl-ester formulation) was purchased from Equateq (Edinburgh, UK). Differentiated neuron-like SH-SY5Y cells plated in 2 mL of culture medium (10 % FBS) in 6-well plates at a density of 2.5×10^4 cells/cm^2 were exposed to HDHA at 5, 10, 20 and 30 μM for 7 h. In addition, differentiated SH-SY5Y cells were exposed to the Aβ peptide (5 μM), disaggregated with NH_4OH (see below), and maintained for 24 h in the presence or absence of either HDHA or DHA (5 and 10 μM). The final concentration of DMSO was always kept below 0.1 %.

Aβ-42 peptide preparation and treatment

The β-Amyloid (1–42) peptide (purity: >95 %) was purchased from Bio Basic Canada Inc (Markham, Canada) and it was dissolved in 1 % (v/v) NH_4OH (Sigma), at a final concentration of 1 mg/mL. It was then submitted to ultrasound for 30 s and 10 W to promote complete disaggregation of the peptide [43]. Finally, it was diluted in PBS (1:10, v:v) immediately prior to use, added to the cell culture medium, and incubated at 37 °C for 24 h to promote peptide aggregation and Aβ-mediated neurotoxicity in SH-SY5Y neuron-like cells [44, 45].

Cell viability (MTT assay)

Cell viability was determined by the MTT (methyl-thiazolyl diphenyl tetrazolium bromide) method [46] as described elsewhere [39]. Briefly, SH-SY5Y cells were plated in 6-well plates at a density of 2.5×10^4 cells/cm^2 and differentiated. After overnight incubation, the cells were treated for a further 24 h with HDHA (from 5 to 30 μM) with or without 5 μM of NH_4OH-disaggregated Aβ peptide. The MTT reagent (Sigma) was diluted in PBS to a final concentration of 0.5 mg/mL and added to the cell culture for 2 h. Mitochondrial dehydrogenases in viable cells reduced the tetrazolium salt, yielding water insoluble colored formazan crystals. Subsequently, the MTT reagent was removed and the formazan crystals were solubilized by adding one volume of DMSO for 5 min. Finally, after gentle shaking, the absorbance at 590 nm was measured spectrophotometrically using a Micro Plate Reader. Background absorbance was determined at 650 nm and subtracted from the 590 nm value.

Protein extraction, quantification, electrophoresis (SDS-PAGE) and immunoblotting

Protein extracts from cells in culture were quantified and western blotting was performed as described previously [39]. Briefly, cells were washed with PBS and harvested with a rubber policeman in protein extraction buffer (50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 % SDS in 10 mM Tris–HCl pH 7.4) containing protease inhibitors (5 mM iodoacetamide and 1 mM PMSF). The cell suspensions were then disrupted by ultrasound and the remaining suspension was mixed with Laemmli's SDS-PAGE loading buffer and boiled for 5 min. The protein concentration was measured using the bicinchoninic acid assay, according to the manufacturer's instructions (Pierce).

Protein samples (30 μg) were resolved by electrophoresis on 8–10 % polyacrylamide gels (SDS-PAGE) in tris–glycine electrophoresis buffer and then transferred to nitrocellulose membranes (GE, Amersharm) that were subsequently blocked with 5 % (w/v) non-fat dry milk in 0.1 % (v:v) Tween-20 PBS (TPBS). When anti-phosphoprotein antibodies were used to probe the membranes, non-fat dry milk was substituted with 1.5 % bovine serum albumin (BSA). The membranes were then incubated overnight at 4 °C with one of the following primary antibodies diluted in TPBS containing 0.5 % (w/v) BSA: anti-IRE1α, anti-CHOP, anti-phospho-Ser51-eIF2α, anti-Bip, anti-Calnexin, anti-PDI, anti-Beclin-1, anti-Atg5, anti-Atg12, anti-Atg7, anti-Atg3, anti-LC3B, anti-Cdk4, anti-Cdk6, anti-DHFR, anti-cyclinD3 (all diluted 1:1,000 and purchased from Cell Signaling), anti-nestin (diluted 1:1,000 and purchased from Abcam) and anti-α-tubulin (diluted 1:10,000 and purchased from Sigma). After removing the primary antibody, the membranes were washed three times for 10 min with TPBS and incubated for 1 h in darkness at room temperature in fresh blocking solution containing IRDye 800CW-linked donkey anti-mouse IgG or IRDye 800CW-linked donkey anti-rabbit IgG (1:5,000; LI-COR Inc.). The membranes were then washed with TPBS and immunoreactivity was detected using an Odissey Infrared Imaging System (LI-COR Inc.). Band intensity was quantified by image analysis, which was processed by integrated optical density using the TotalLab v2005 software (*Nonlinear Dynamics*, UK) and normalized using α-tubulin as a reference housekeeping protein.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.01 (*GraphPad Software Inc*., USA). Unless indicated, data were expressed as the mean ± SEM from at least three independent experiments (*n*). Statistical comparisons between two groups of data were performed using unpaired *t* test. For comparison between several groups, we used one-way ANOVA followed by Bonferroni's post hoc test. The symbols below indicate the level of significance with

respect to the indicated control condition: $^{*,\#}p < 0.05$; $^{**,\#\#}p < 0.01$; $^{***,\#\#\#}p < 0.001$.

Results

HDHA induces concentration-dependent UPR and apoptosis in SH-SY5Y neuron-like cells

Molecular chaperones are key elements in the ER stress response/UPR [47]. Therefore, we first studied the effect of HDHA on the three main known chaperones: Bip (also named Grp78 or Hsp70), a classic chaperone implicated in the correct folding of a wide range of proteins; Protein Disulfide Isomerase (PDI), a folding enzyme of disulfide-bonded proteins; and calnexin (CNX), a specific chaperone that mediates folding of glycoproteins. In quantitative immunoblots, we studied the effect of HDHA (5–30 μM; 7-h incubation) on the expression of these chaperones in neuron-like SH-SY5Y cells. The presence of HDHA induced a concentration-dependent increase of Bip protein as compared with the control (untreated) cells (Fig. 1a). Hence, HDHA enhanced the UPR in this neuron model. By contrast, CNX expression was only significantly reduced at higher HDHA concentrations (20 and 30 μM: Fig. 1a), a down-regulation that might lead to cell death through apoptosis, as described previously [48].

Subsequently, we focused on ER stress sensors and their associated signaling pathways. Of the most commonly studied ER sensors: PERK, IRE1 and ATF6, PERK-eIF2α [49] and IRE1 [50] have been previously associated with the activation of autophagy. PERK is a sensor that mediates eiF2α phosphorylation at Ser51 once activated and exposure to HDHA induced a concentration-dependent Ser51 phosphorylation of the eIF2α transcription factor in SH-SY5Y cells (Fig. 1b, left panel). Indeed, the increased eIF2α phosphorylation was significant even at the lowest concentration of HDHA tested (5 μM), and it was gradually enhanced to reach a more than threefold increase at 30 μM (Fig. 1b, right panel). Thus, it would appear that the effect of HDHA on UPR is mediated through PERK activation. Indeed, no differences in IRE1α expression were observed

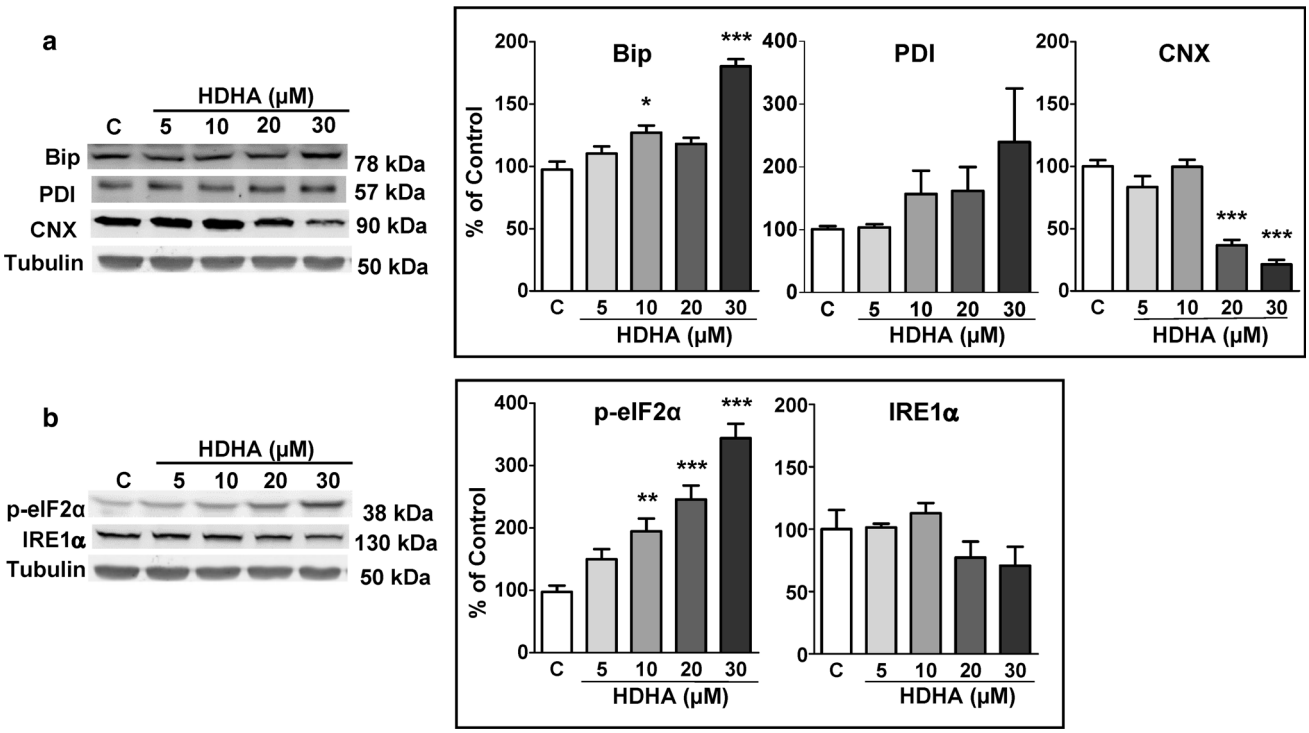


Fig. 1 HDHA induces concentration-dependent activation of the UPR in SH-SY5Y neuron-like cells after a 7-h exposure. **a** Representative western blot showing Bip, PDI and CNX expression in SH-SY5Y cells maintained in the presence of increasing concentrations of HDHA (5–30 μM). Bip expression increased when exposed to 10 and 30 μM HDHA compared with the untreated control, while CNX was not affected at lower doses (5–10 μM) but decreased strongly at higher concentrations of HDHA (20–30 μM). **b** Representative western blot showing eIF2α phosphorylation at Ser51 and IRE1α expression in SH-SY5Y cells maintained in the presence of increasing doses of HDHA (5–30 μM). Phosphorylation of eIF2α increased in a concentration-dependent manner and was highest at 30 μM, while no differences were observed in IRE1α at any of the concentrations of HDHA tested. Immunoblot values are expressed relative to the untreated control (*C*). Bars represent the mean ± SEM. The statistical analysis was performed by ANOVA and the asterisks indicate a significant effect of the treatment compared with the untreated control (*C*): $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

after exposing these neuron-like cells to HDHA (Fig. 1b), suggesting that PERK-eIF2α is the principal arm of the UPR activated by HDHA in our cell model.

Finally, to test whether the HDHA-induced UPR in SH-SY5Y cells is pro-survival or pro-apoptotic, we studied the expression of the ER-stress-mediated apoptosis marker CHOP (C/EBP homologous protein transcription factor, also named GADD153). Under chronic ER stress, CHOP expression can be induced by the three aforementioned arms of the UPR (the pathways led by PERK, IRE1 and ATF6), leading to apoptosis via Bcl-2 down-regulation [51]. Interestingly, exposure to HDHA only induced CHOP expression at high concentrations (20 and 30 μM), whereas the CHOP protein was undetectable in differentiated SH-SY5Y cells exposed to lower concentrations of HDHA (5 and 10 μM, Fig. 2a). To assess the relationship between CHOP-induced expression and neuronal cell death, we tested cell viability in cultures exposed to HDHA. As expected, cell viability increased modestly but significantly in the presence of lower HDHA concentrations (5 μM) [41], whereas higher concentrations (20 and 30 μM) of HDHA induced a marked decrease in the number of surviving cells compared to the untreated controls (Fig. 2b). This decrease in cell viability in the presence of HDHA coincided with CHOP upregulation and CNX downregulation, indicating that cell death may be caused by apoptosis. In this context, a mild UPR (characterized by up-regulation of Bip and followed by PERK activation and the phosphorylation of eIF2α) was present at the lower concentrations of HDHA used (5 and 10 μM), without affecting CHOP or CNX expression. Consequently, it is reasonable to consider that concentrations of HDHA below 20 μM may be "therapeutic" to SH-SY5Y neuron-like cells under these experimental conditions.

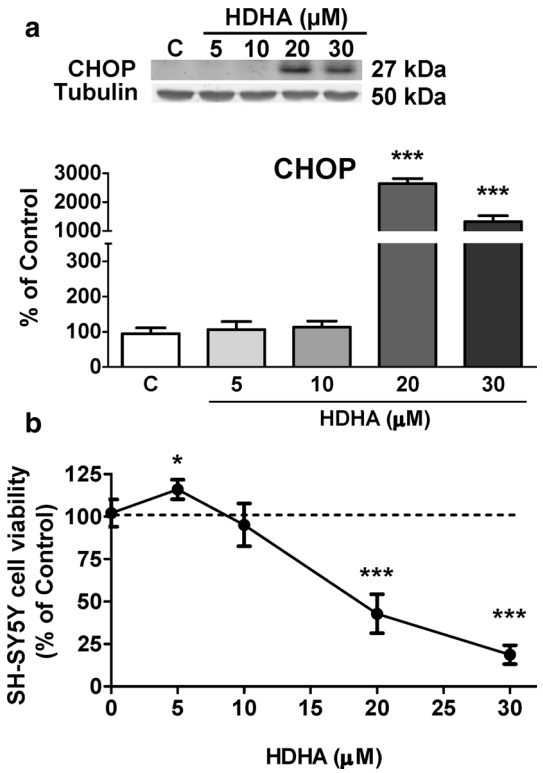


Fig. 2 CHOP-induced expression correlates with neuronal cell death in HDHA-treated SH-SY5Y neuron-like cells in a concentration-dependent manner. **a** Representative western blot of CHOP expression induced after 7 h, showing the increase in CHOP expression induced at higher concentrations (20 and 30 μM) of HDHA in differentiated SH-SY5Y cells, while it remained unaltered at lower concentrations (5 and 10 μM). **b** MTT assay showed unaltered cell viability after a 24-h exposure to lower concentrations of HDHA (5 and 10 μM), which decreased dramatically in the presence of 20 μM HDHA and even further with 30 μM HDHA. Immunoblot and MTT values are expressed relative to the corresponding untreated controls (*C*). The bars represent the mean ± SEM. The statistical analysis was performed by ANOVA and the asterisks indicate a significant effect of the treatment compared with the untreated control (*C*): $***p < 0.001$

Autophagy is up-regulated by HDHA in differentiated SH-SY5Y cells

Like UPR, autophagy is triggered by cells to clear misfolded or aggregated proteins that have accumulated intracellularly, thereby compromising cell survival. For this reason, autophagy has been assigned a neuroprotective function. However, like ER stress, sustained activation of autophagy can also lead to neuronal death [52]. We characterized some key markers of autophagy in immunoblots to determine if autophagy is induced by HDHA in SH-SY5Y neuron-like cells (see Fig. 3), treating cells for 7 h with only sub-lethal (therapeutic) concentrations of HDHA (5 and 10 μM: see Fig. 2). First, we assessed beclin-1 expression, a protein involved in the initiation of autophagy (phagophore nucleation) along with the other proteins contained in the Beclin1-Vps34 complex [53, 54]. A modest but significant increase in beclin-1 was detected after exposure to 10 μM HDHA compared with the untreated control (Fig. 3a, right panel). Hence, HDHA appears to be able to trigger autophagy at concentrations that do not induce CHOP-mediated apoptosis in SH-SY5Y neuron-like cells.

We next assessed if elongation and maturation of immature AVs is stimulated by HDHA, as well examining the autophagy-related gene products (Atgs) involved in the ubiquitin-like conjugation that participates in these processes. On the one hand, we evaluated Atg5 and Atg12 expression, both proteins that form a complex with Atg16 located in the immature AV membrane and that promotes the elongation of primary vesicle structures [55, 56]. Both Atg5 and Atg12 are more strongly expressed by SH-SY5Y neuron-like cells after exposure to HDHA (Fig. 3a, left

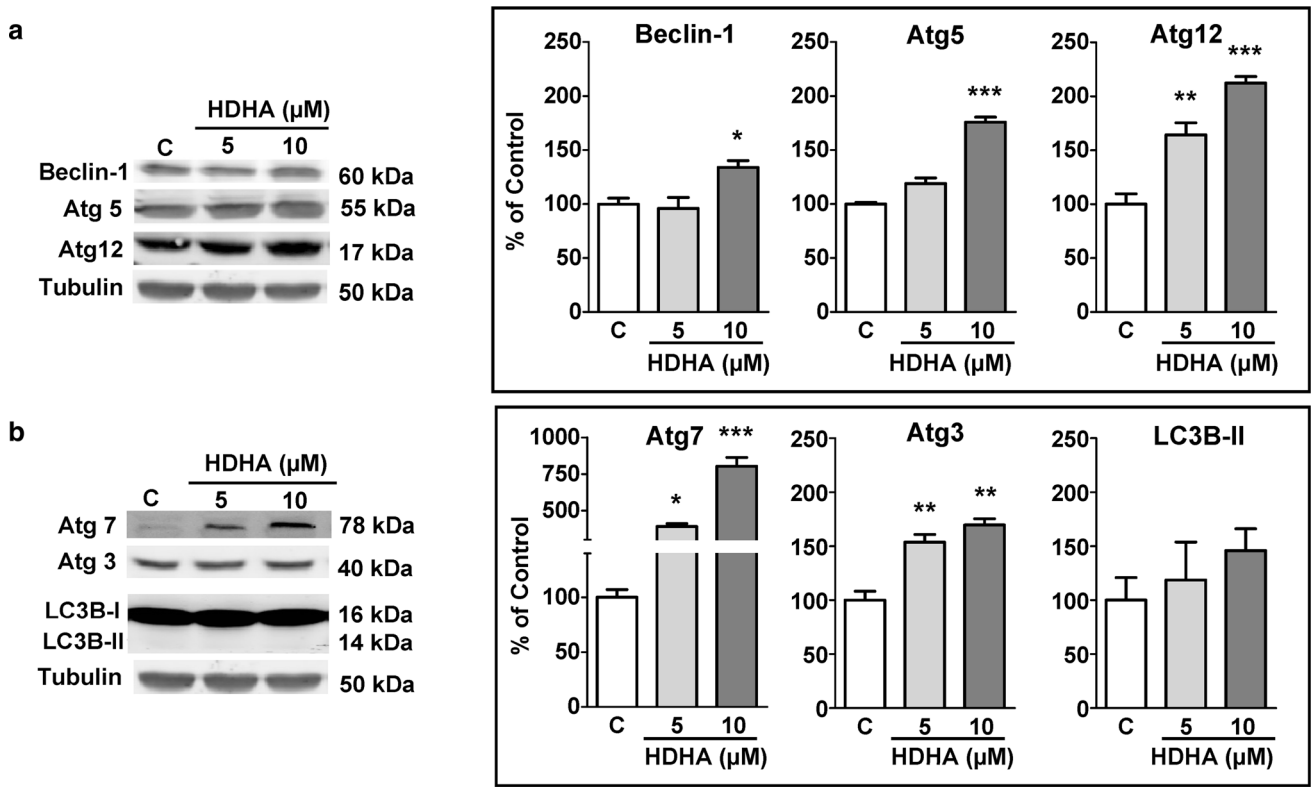


Fig. 3 Autophagic response in SH-SY5Y neuron-like cells exposed to therapeutic concentrations of HDHA for 7 h. **a** Representative western blot showing Beclin-1, Atg5 and Atg12 expression in SH-SY5Y cells. The marker of autophagy initiation, beclin-1, as well as Atg5 and Atg12, were up-regulated in the differentiated SH-SY5Y cells exposed to HDHA for 7 h. **b** Representative western blot showing Atg7, Atg3 and LC3B expression in SH-SY5Y cells. The expression of Atg proteins involved in AV maturation, Atg7 and Atg 3, was also enhanced after a 7-h incubation with either 5 or 10 μM of HDHA, however LC3B-II was not significantly affected when compared to the control conditions. The immunoblot values are expressed relative to the untreated control (*C*). The bars represent the mean ± SEM. The statistical analysis was performed by ANOVA and the asterisks indicate a significant effect of the treatment compared to the untreated control (*C*): $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

panel) and indeed, therapeutic concentrations of HDHA (5 and 10 μM) up-regulated Atg5 and Atg 12 in a concentration-dependent manner (Fig. 3a, right panel). Similarly, the role of microtubule-associated protein 1 light chain 3 (LC3) in AV maturation was also studied as conjugating the cytosolic form of LC3 (LC3-I) to phosphatidylethanolamine (PE) generates the membrane-anchored form (LC3-II) and this is a key step in the maturation of AVs in mammalian cells [57, 58]. This process depends on several proteins including Atg7, Atg3 and the complex consisting of Atg5, Atg12 and Atg 16. In this context, HDHA induced a significant concentration-dependent increase of Atg7 and Atg3 in SH-SY5Y neuron-like cells (Fig. 3b, left panel). These changes were especially relevant for Atg7, which increased fourfold with 5 μM and ninefold with 10 μM HDHA with respect to control (untreated) cells (Fig. 3b, right panel). Interestingly, Atg7 is also involved in Atg5-Atg12 complex formation, suggesting that HDHA-mediated upregulation of Atg7 may stimulate AV maturation through both pathways, favoring Atg5-Atg12-Atg16 complex formation and LC3 conjugation to lipid membranes. Finally, we investigated LC3-II expression under the same conditions in which beclin-1 and Atg proteins were tested (Fig. 3b, left panel). Despite the increase in beclin-1, Atg5, Atg12, Atg7 and Atg3 in the presence of HDHA, the levels of LC3-II did not differ significantly in these cells under the same experimental conditions (5 and 10 μM HDHA: Fig. 3b, right panel). In this sense, we previously showed that HDHA failed to modulate LC3 expression in N2a neuroblastoma cells [41]. This apparent discrepancy between the HDHA-mediated activation of autophagy and the lack of an effect on LC3-II could indicate that LC3-II levels are more representative of AV density than of the LC3-I to LC3-II conversion [59].

HDHA-induced autophagy upon Aβ insult correlates with improved survival of SH-SY5Y neuron-like cells

We found that a relatively short exposure to HDHA at low (therapeutic) concentrations induces mild UPR and

relevant activation of autophagy, which involves AV initiation and maturation, whereas exposure to higher concentrations (20 and 30 μM) led to neuron cell death probably mediated by apoptosis via CHOP (and perhaps also CNX: see Fig. 2). Thus, we further evaluated the autophagy response of SH-SY5Y differentiated cells to therapeutic concentrations of HDHA in the presence of a high concentration of Aβ (5 μM) and after a longer exposure (24 h). This in vitro model in part mimics the neurotoxic environment that neuronal cells may experiment in the AD brain. In these studies, the Aβ peptide was added in its monomeric form and incubated at 37 °C for 24 h in the same medium as HDHA in order to promote aggregation into soluble oligomers and/or insoluble aggregates that are thought to be neurotoxic [44, 45]. Furthermore, we used this model to compare the effects of HDHA and its non-hydroxylated form (DHA) on autophagy. In this context, when we first assessed the marker of the initiation of autophagy, beclin-1, this protein was significantly upregulated in Aβ-stimulated cells exposed to HDHA (10 μM) when compared with the control condition (also stimulated with Aβ: Fig. 4). By contrast, the non-hydroxylated form of DHA had no significant effect on Beclin-1 levels (Fig. 4a).

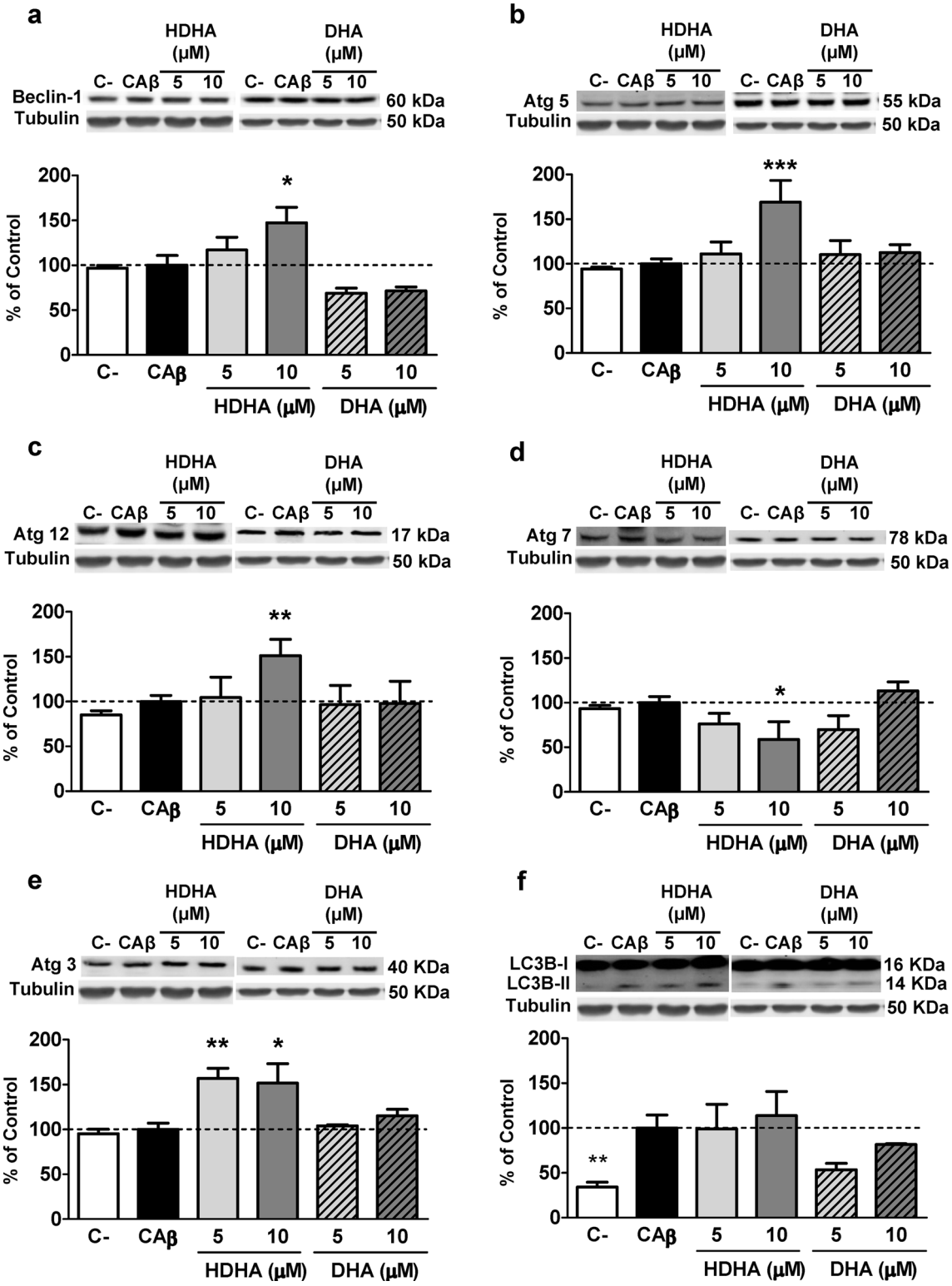
We next evaluated the expression of the Atg proteins involved in AV maturation (Atg5, Atg12, Atg7, Atg3 and LC3-II) and we observed similar effects under these new experimental conditions to those described previously (see Fig. 3). Atg5 and Atg12 levels increased significantly on exposure to HDHA (10 μM) but they were not modified at 5 μM, and as shown previously for Beclin-1, nor did DHA have a significant effect on Atg5 or Atg12 expression compared with the control untreated cells (Fig. 4b and c). As a part of the enzymatic mechanism mediating conjugation of LC3-I to PE, we also studied the expression of Atg3 and Atg7 in cells exposed to Aβ and HDHA. HDHA induced an increase in Atg3 at 5 and 10 μM (Fig. 4e), while again DHA failed to produce a significant effect on SH-SY5Y neuron-like cells under these conditions (Fig. 4e). Surprisingly, HDHA 10 μM diminished Atg7 levels from those in control untreated cells (Fig. 4d), in contrast to the strong induction of Atg7 that had been seen in the absence of Aβ (Fig. 3b, right panel). Like other markers of autophagy tested, Atg7 was not modulated by the non-hydroxylated form of DHA (Fig. 4d). Finally, we also tested LC3-II expression in these cultures, which was up-regulated in the presence of Aβ alone (Fig. 4f: see CAβ vs. C-). Since the expression of other autophagy markers tested was not modified by Aβ (Beclin-1 and Atg proteins), this increase in LC3-II could be due to the accumulation of AVs as a result of Aβ stimulation [60]. However, exposure to HDHA or the non-hydroxylated form of DHA did not modulate the LC3-II levels (see Figs. 4f and 3b).

Finally, we tested cell survival of SH-SY5Y cells in the presence of Aβ (10 μM to enhance the neurotoxic effects of this neurotoxic peptide [41]) and HDHA or DHA. Our results showed a loss of neuron-like cell viability when exposed to Aβ (CAβ) as compared with the negative control (not exposed to Aβ) (35.68 ± 6.85 %: Fig. 5a, white-filled bars). While exposure with DHA was unable to protect Aβ-induced cell death, HDHA significantly increased cell viability by 15.54 ± 5.22 % as compared with the control treated with Aβ alone (CAβ; see Fig. 5a, white-filled bars) which means that HDHA was able to prevent 43.55 % of total Aβ-induced cell death (Fig. 5a, gray-filled bars). Since these treatments were performed using 10 μM HDHA, a link may be proposed between HDHA-induced autophagy and improved cell survival following exposure to toxic Aβ. In addition, this association is supported by earlier data showing a clear induction of tau protein phosphorylation (Ser202/Thr205; AT8 epitope) in the presence of Aβ that was markedly inhibited by exposing SH-SY5Y neuron-like cells at the same concentration of HDHA (10 μM: Fig. 5b) [41].

Discussion

Differentiated SH-SY5Y cells represent an appropriate model to study neurodegenerative diseases like Parkinson and AD [42, 61]. In the present work, we studied the effects of the 2-hydroxyl-derivative of DHA, HDHA, on the UPR and autophagy pathways in this cell model. Previously, we demonstrated that HDHA restores cognitive performance in an animal model of AD (5xFAD mice). In these mice: (a) cognitive performance following HDHA administration was similar to that of healthy control mice [40]; (b) HDHA significantly up-regulated hippocampal neuronal cell proliferation [40]; (c) HDHA modified brain lipid membrane composition, enriching it in PE carrying long PUFAs that promoted the formation of membrane liquid-disordered structures [41, 62]; and (d) HDHA decreased the total amyloid load and Ser202 tau phosphorylation [40, 41]. This evidence clearly shows the potential therapeutic effect of HDHA against AD, although the molecular mechanism connecting all these events is yet to be fully understood.

In this work, we used the SH-SY5Y neuron-like cell model to explore whether HDHA mediates its therapeutic effects by up-regulating UPR and autophagy signaling. We found that exposure to HDHA induces the expression of key proteins involved in the UPR in a concentration-dependent manner, such as the molecular chaperone Bip, as well as the PERK sensor-dependent phosphorylation of eIF2α. The induction of the UPR by HDHA was biphasic, with a mild ER stress response at low concentrations (<20 μM) and a stronger ER stress response characterized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C- CAβ 5 10 C- CAβ 5 10
LC3B-I
LC3B-II
Tubulin
16 KDa
14 KDa
50 KDa
**
C- CAβ 5 10 5 10
HDHA (μM) DHA (μM)

◀**Fig. 4** Autophagy response in SH-SY5Y neuron-like cells exposed to therapeutic concentrations of HDHA in the presence of Aβ (5 μM) for 24 h. Representative western blot showing Beclin-1 (**a**), Atg5 (**b**), Atg12 (**c**), Atg7 (**d**), Atg3 (**e**) and LC3B (**f**) expression in the SH-SY5Y cells. **a** The autophagy initiation marker beclin-1 was up-regulated in Aβ-stimulated cells after HDHA treatment when compared to the Aβ-treated controls (CAβ), as were some of the Atg proteins involved in AV maturation [Atg5 (**a**), Atg12 (**c**), and Atg3 (**e**)]. Less Atg7 (**d**) was expressed after HDHA treatment (10 μM) than in the control cells (CAβ), while LC3B-II (**f**) was not modulated by HDHA. The non-hydroxylated form of DHA failed to show any significant effect on Atg proteins in SH-SY5Y neuron-like cells under these experimental conditions. Immunoblot values are expressed relative to the Aβ-treated controls (CAβ) and each bar diagram shows the mean ± SEM. The statistical analysis was performed by ANOVA of all the conditions treated with the same drug and the asterisks indicate a significant difference compared with the Aβ-treated controls (CAβ): $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Comparison of two different conditions (C- vs CAβ) was performed by unpaired *t* test: $** p < 0.05$

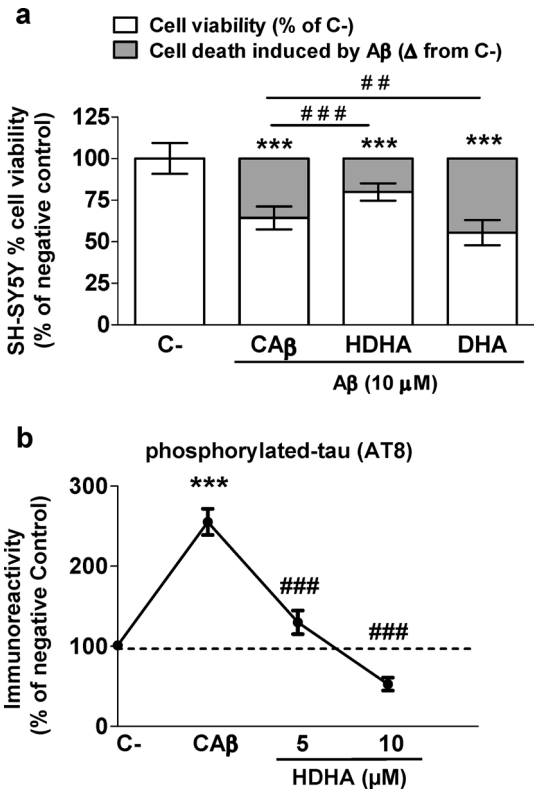

Fig. 5 Increased neuronal survival correlates with the down-regulation of tau phosphorylation in HDHA-treated SH-SY5Y neuron-like cells. **a** The MTT assay demonstrated a reduction in cell viability after 24 h in the presence of Aβ (CAβ) when compared to the untreated control cells (C-). The viability of these neuronal cells was recovered when they were exposed to Aβ in the presence of HDHA (10 μM) but not DHA (10 μM). **b** In Western blots of phospo-tau in neuron-like differentiated SH-SY5Y cells there was a strong induction of tau phosphorylation at the AT8 epitope (Ser202/Thr205) after Aβ stimulation (10 μM). Whereas the presence of HDHA (5 μM) partly reduced Aβ-mediated tau phosphorylation and it was completely abolished in the presence of 10 μM HDHA (adapted from [41]). Values are expressed relative to untreated controls (C-) and the data was plotted as the mean ± SEM. The statistical analysis was performed by ANOVA and significant differences were detected with respect to the untreated control: $***p < 0.001$; difference to Aβ-treated control (CAβ): $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$

by CHOP upregulation and cell death at higher concentrations (>20 μM). Moreover, the lower concentrations of HDHA, which have been considered here as "therapeutic concentrations" (5 and 10 μM), also up-regulated beclin-1 and Atg expression, proteins involved in the initiation of autophagy and AV maturation, respectively. A similar induction of autophagy was also found in conditions of Aβ-mediated neurotoxicity and cells were significantly protected from Aβ-induced death by concomitant exposure to therapeutic concentrations of HDHA but not by the non-hydroxylated DHA. Consequently, it might be hypothesized that the HDHA-promoted UPR and autophagy signaling plays a central role in reducing Aβ production and tau phosphorylation, thereby enhancing neuronal cell viability.

Our previous results suggested that HDHA initially acts at the membrane, modifying membrane lipid composition and structure by normalizing lipid levels at the bilayer, mainly impeding the dampening in PE and PUFAs associated with AD [41]. Indeed, treatment with DHA (or other PUFAs) up-regulates key mediators of UPR in several studies, leading to cell cycle arrest and apoptosis in cancer cell lines. This effect was proposed to take place downstream perturbations in Ca^{2+} homeostasis and oxidative stress, unavoidably driving cancer cells towards apoptosis [63, 64]. Interestingly, DHA-mediated UPR in such cancer cell lines was characterized by a time- and dose-dependent up-regulation of eIF2α phosphorylation and CHOP expression, similarly to that shown here (Fig. 1) [64, 65], suggesting that HDHA might share the same molecular mechanisms with DHA. Although such DHA effects on the UPR would depend on the species and cell type under study [66], it is noteworthy that we have clearly detected two different effects in the range of HDHA concentrations evaluated. The first effect is a cell death provoked by 20–30 μM HDHA which was paralleled by a strong induction of CHOP expression and CNX downregulation suggesting cell death via apoptosis. Nevertheless, it should not be ruled out that prolonged suppression of protein synthesis as consequence of elevated eIF2α phosphorylation would also contribute to SH-SY5Y cell death. The second effect is the mild UPR and autophagy that were triggered by therapeutic concentrations of HDHA. Hence, such therapeutic concentrations may allow productive folding to occur more efficiently in SH-SY5Y cells without promoting cell death. In this context, these results are in agreement with previous data showing that therapeutic

doses of HDHA for a transgenic mouse model of AD are in the range of 15–50 mg/kg [40, 41]. Interestingly, the Irwin test [67] in the same mouse strain at concentrations as high as 200 and 500 mg/kg (daily) failed to detect clinical alterations, nor was there any weight loss after a 3-month treatment with a daily dose of 200 mg/kg (see online resources 2 and 3). These evidences show lack of side-effects of HDHA in mice even at doses tenfold over the therapeutic range. Hence, the therapeutic effects observed in HDHA-treated mice might be supported at least in part by the mild UPR and autophagy activation as shown now in cell cultures when using therapeutic concentrations of HDHA.

The three known UPR signaling arms are PERK-eIF2α, IRE1 and ATF6, and they have each been linked to the induction of autophagy [49, 50, 68]. In this sense, we showed that HDHA induced a dose-dependent up-regulation of eIF2α phosphorylation in SH-SY5Y differentiated cells. The PERK-eIF2α pathway can modulate autophagy through eIF2α phosphorylation-dependent Atg12 up-regulation in response to polyQ protein accumulation [49]. Consequently, the expression of autophagy-related genes by the PERK-eIF2α arm of the UPR could be one of the mechanisms connecting both these events. However, we found here that HDHA increased the expression of most Atg proteins and not only Atg12, such that HDHA itself might up-regulate the autophagy-related genes even though stimulating the PERK-eIF2a pathway might be responsible for the increase of Atg12 expression in SH-SY5Y cells. Indeed, we also detected the up-regulation of beclin-1 levels by HDHA, a key part of the Beclin1-Vps34 complex involved in initiating autophagy [53, 54]. This result strongly supports a role for HDHA in the induction of autophagy irrespective of its effects on UPR induction. Conversely, and in line with evidence that DHA induces the UPR/apoptosis in cancer cell lines, apoptosis linked to the induction of autophagy by DHA has been described in cancer cell lines [69, 70]. However, again we showed here that autophagy was induced by sub-lethal therapeutic concentrations of HDHA (5 and 10 μM), with no CHOP-induced apoptosis detected.

How HDHA (or DHA) induces UPR and autophagy is ill known. Slight chemical modifications in key atoms of the molecular structure are supposed to modify the biological properties of DHA, i.e. by increasing affinity for certain receptors such as PPAR-γ [71]. That is the case of natural DHA-hydroxyl derivatives such as NPD1 (neuroprotectin D1) that binds to this receptor to exert its neuroprotective activity whereas non-hydroxylated DHA shows no/minor effect mediated by PPAR-γ [71]. In addition, in the case of HDHA, it should not be ruled out the effect as consequence of increased half-life at biological membranes because of the hydroxylation located at the α-carbon that potentially inhibits fatty acid β-oxidation [72]. In this context, it is noteworthy that DHA-mediated PPAR-γ activation has been directly related to autophagy stimulation [73]. Consequently, it might be hypothesized that higher affinity of HDHA for PPAR-γ might explain the higher effect of this hydroxyl derivate in stimulating autophagy whereas the native form of DHA showed no effect at the therapeutic concentrations used in the present work in SH-SY5Y neuron-like cells. Other mechanisms explaining the mechanism of action of HDHA have been focused on the lipid membrane composition and structure [41, 62], although research on these molecular mechanisms is still ongoing.

How stimulating the UPR and autophagy can promote neuronal cell survival is an issue that has received much attention [1, 74]. In the context of AD, Bip can bind to nascent APP and facilitate its correct folding, which would limit Aβ42 peptide production and accumulation in the cell, thereby promoting neuronal survival [75]. However, once the Aβ peptide has already formed, chaperones would not be able to eliminate it and other mechanisms such as autophagy must be activated. In the same context, activation of autophagy may confer neuroprotection to neuronal cells since this pathway has been directly linked to the processing and clearing of APP and its metabolites, such as Aβ peptides that accumulate in AVs in the AD patient's brain and in animal models of AD [26, 28]. We show that HDHA improves the survival of cells subjected to an Aβ insult, which suggests that apart from the role of the UPR and autophagy in alleviating the intracellular production and/or accumulation of APP/Aβ, additional mechanisms may protect these cells. In this context, Aβ stimulation induces marked tau Ser 202/Thr205 hyperphosphorylation in SH-SY5Y cells [41] and intracellular accumulation of hyperphosphorylated tau would interfere with microtubule-associated vesicle transport, compromising cell viability [76]. In this study, we have documented the relationship between the dampening of tau phosphorylation and increased neuronal survival in HDHA-treated SH-SY5Y neuron-like cells. Furthermore, the inhibition of autophagy has been correlated with increased hyperphosphorylated tau protein [77]. Consequently, it would be expected that stimulating autophagy with HDHA would decrease tau phosphorylation in SH-SY5Y cells. Although this hypothesis would explain the increased cell viability after HDHA treatment in Aβ-insulted SH-SY5Y cells, further research is needed to fully understand this process.

In summary, we have investigated the role of the UPR and autophagy in the mechanism of action of HDHA. The results shed light on the possible action of this drug as a promising therapeutic alternative for the AD treatment.

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