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The omega-3 fatty acid, DHA, decreases neuronal cell death in association with altered zinc transport

Cenk Suphioglu [a,*], Damitha De Mel [a], Loveleen Kumar [a], Nadia Sadli [a], David Freestone [a], Agnes Michalczyk [a], Andrew Sinclair [b,c], M. Leigh Ackland [a]

[a] School of Life and Environmental Sciences, Deakin University, 221 Burwood Highway, Burwood, VIC 3125, Australia
[b] School of Exercise and Nutrition Sciences, Deakin University, 221 Burwood Highway, Burwood, VIC 3125, Australia
[c] School of Medicine, Deakin University, 221 Burwood Highway, Burwood, VIC 3125, Australia

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ABSTRACT

Docosahexaenoic acid (DHA) is the major polyunsaturated fatty acid in neuronal cell membranes. We hypothesize that DHA induces a decrease in neuronal cell death through reduced ZnT3 expression and zinc uptake. Exposure of M17 cells to DHA-deficient medium increased the levels of active caspase-3, relative to levels in DHA-replete cells, confirming the adverse effects of DHA deficiency in promoting neuronal cell death. In DHA-treated M17 cells, zinc uptake was 65% less and ZnT3 mRNA and protein levels were reduced in comparison with DHA-depleted cells. We propose that the neuroprotective function of DHA is exerted through a reduction in cellular zinc levels that in turn inhibits apoptosis.

1. Introduction

The omega-3 fatty acid, alpha-linolenic acid (ALA), is an essential nutrient that cannot be synthesized by mammals [1]. The mammalian brain is rich in a metabolite of ALA known as DHA (docosahexaenoic acid, 22:6n-3); this fatty acid is the main polyunsaturated fatty acid (PUFA) in the brain where it makes up approximately 3% of the dry weight of the brain [2]. DHA has many diverse functions at the cellular level including effects on membrane fluidity, regulation of enzymes and ion channels and in signal transduction [3]. Evidence from cell culture and animal models suggests that n-3 PUFA supplementation attenuates β-amyloid deposition, a hallmark of Alzheimer's disease [4–9]. Furthermore, mechanistic studies have demonstrated that DHA promotes neuronal survival via Akt [10], Bcl-2 [11] and brain derived neurotrophic factor [12] signalling pathways. Collectively, these studies along with post-mortem studies demonstrating lower brain DHA in Alzheimer's patients [8,13,14] provide mechanistic support for the existence of a relationship between n-3 PUFA intake and Alzheimer's disease risk.

In a previous study of dietary DHA deficiency in rats, we found a novel link between DHA deficiency and altered brain zinc homeostasis. Adult animals on a DHA-deficient diet had decreased plasma zinc levels, altered brain zinc distribution and increased expression of a member of the zinc transporter family, ZnT3 (SLC30A3) in the brain [15]. These findings are consistent with well-established links between zinc homeostasis and lipid metabolism [16–19]. A common feature of both DHA and zinc is their capacity to influence neuronal cell death. DHA prevents apoptotic death induced by serum deprivation [18,19] while zinc is a known potent toxic cation mediating neuronal injury [20] and apoptotic cell death [21]. The molecular basis of the link between DHA and zinc remains unknown. Therefore, the aim of the current study was to explore the link between the omega-3 fatty acid DHA and zinc metabolism, specifically in relation to cell death through apoptosis.

2. Materials and methods

2.1. Cell culture

The human neuroblastoma cell line M17 was grown at 37 °C in a humidified atmosphere in the presence of 5.0% carbon dioxide, as monolayer cultures in 75 cm^2 disposable plastic flasks (Nunc, Roskilde, Denmark), maintained in 10 ml of Opti-MEM media

* Corresponding author. Address: School of Life and Environmental Sciences, Faculty of Science and Technology, 221 Burwood Highway, Burwood, VIC 3125, Australia. Fax: +61 3 9251 7328.
E-mail address: cenk.suphioglu@deakin.edu (C. Suphioglu).

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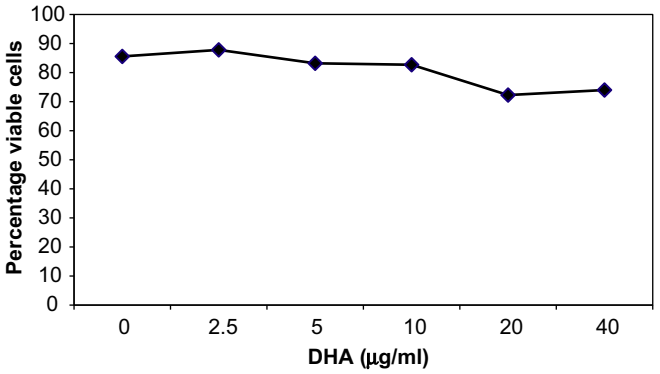

Fig. 1. Cell viability plot of the M17 human neuronal cell line. M17 cells cultured in the absence and presence of different DHA concentrations (μg/ml) were stained with Trypan blue and the percentage of viable cells was determined.

(a modified MEM Eagle's media) with heat-inactivated 2.5% foetal bovine serum (FBS) supplementation. At ~90% confluence, M17 cells were harvested or passaged using 0.025% trypsin/EDTA.

M17 cells were seeded at a density of 1×10^6 cells/75 cm^2 flask and grown to confluence then exposed to media supplemented with DHA (Sigma–Aldrich, MO, USA; 2.5, 5, 10, 20 and 40 μg/ml final concentration). The DHA-containing media had been pre-incubated overnight at 37 °C to allow the DHA to conjugate with media proteins and allow delivery into cells. After 2 days of incubation, cells were harvested, centrifuged at 1000×*g* for 5 min and pellet resuspended in PBS. Each sample was then divided into 3 aliquots, centrifuged at 14 000×*g* for 5 min and cell pellets stored at −80 °C until needed for analysis.

2.2. Fatty acid analysis

M17 cells were cultured with and without DHA (as described above) and subjected to fatty acid analysis. Cellular phospholipids were isolated by thin layer chromatography and the phospholipid fatty acids were then converted to fatty acid methyl esters for determination of the DHA content by gas chromatography (GC) following our established techniques [22].

2.3. ^{65}Zinc influx experiments

Zinc influx experiments were performed as previously described [23]. Briefly, M17 cells were seeded (in triplicate) in 6 well plates (Nunc, Roskilde, Denmark) at 5×10^5 cells/well and grown in the absence or presence of DHA (20 μg/ml) for 2 days, as described above. Spent media was removed and replaced with fresh media containing ^{65}Zinc and 5 μM $ZnCl_2$, in the absence or presence of DHA (20 μg/ml) and incubated at 37 °C for 0, 2, 5, 10, 20 or 60 min. After incubation, cells were gently washed and harvested as described above. Harvested cells were pelleted at 8000×*g* for 1 min and radioactive signal was measured with a Perkin–Elmer 1480 automatic gamma counter. Total DNA of cell pellets were determined with a Hoechst 33258 dye (Sigma–Aldrich) and measured by VersaFluor fluorometer (Biorad, NSW, Australia). Herring sperm DNA (Sigma–Aldrich) was used as the DNA standard. The specific activity of the zinc was determined and zinc uptake was expressed as pmol Zn/μg DNA.

2.4. Real-time PCR analysis

Total RNA was isolated from cell pellets using RNeasy Mini-kit (Qiagen, VIC, Australia) according to manufacturer's instructions, along with DNA-free treatment (Ambion, TX, USA) to remove genomic DNA (see Supplementary data 1 and Supplementary Table 1).

2.5. Western blot analysis

Lysis buffer (1% sodium dodecyl sulfate, 10 mM Tris–hydrochloric acid, pH of 6.8, containing one Mini EDTA-free protease inhibitor cocktail tablet (Roche Applied Science, NSW, Australia) per 10 ml) was added to cells in flasks. Cells were disrupted by passing through a 21-gauge needle 10 times and sonicated (40% power output, 30% duty cycle) on ice, three times for 15 s with 30 s break between each sonication, by using a Microsone Ultrasonic cell disrupter (Misonix Incorporated, NY, USA). Samples were then centrifuged at 14 000×*g* for 20 min at 4 °C and stored in small aliquots at −80 °C until needed for analysis.

Quantification of protein concentration, gel electrophoresis and Western blot analysis was carried out as described previously [24] and in Supplementary data 2.

2.6. ZnT3 antibody

A synthetic peptide was prepared, consisting of the N-terminal sequence of the hZnT3 gene (VSPRDRGGAGGSLRL) (Mimotopes, Australia) was used to raise an antibody to the human protein as described in Supplementary data 3.

2.7. Immunofluorescence experiments

Immunofluorescence was performed as previously described [25]. See Supplementary data 4.

3. Results

3.1. Proliferation and viability of cultured M17 cells was not affected by DHA concentrations up to 40 μg/ml over 2 days

Total viable cell counts were performed on M17 cells grown in medium enriched with DHA concentrations from 0 to 40 μg/ml for 2 days (Fig. 1). In M17 cells treated with 40 μg/ml DHA for 2 days, the total viable cell count was more than 75%, which was similar to that of the untreated cells (~85%), indicating no direct toxic effect of DHA at 40 μg/ml.

3.2. Cultured M17 cells incorporated DHA

Gas chromatograph analysis indicated that following treatment with DHA (20 μg/ml), M17 cells incorporated DHA from the cell culture medium into the cell membrane phospholipids to a level 4 times that of untreated cells (data not shown), confirming the successful delivery of DHA to the cells. Gas chromatograph analysis indicated that undetectable DHA was present in normal (DHA-deficient) culture medium.

3.3. DHA reduced protein levels of caspase-3, an end marker of apoptosis

M17 cells exposed to 20 μg/ml DHA for 2 days showed a marked reduction in caspase-3 protein levels compared with untreated cells (Fig. 2A) and normalisation to the β-actin housekeeping gene (Fig. 2B) indicated a greater than 66% reduction in caspase-3 protein levels when compared with the untreated cells (Fig. 2C).

3.4. The cellular uptake mechanisms of DHA and zinc are not linked

To establish if DHA and zinc shared a common cellular uptake transport process such as a co-transporter, we measured cellular accumulation of radiolabelled zinc (^{65}Zn) in the presence and

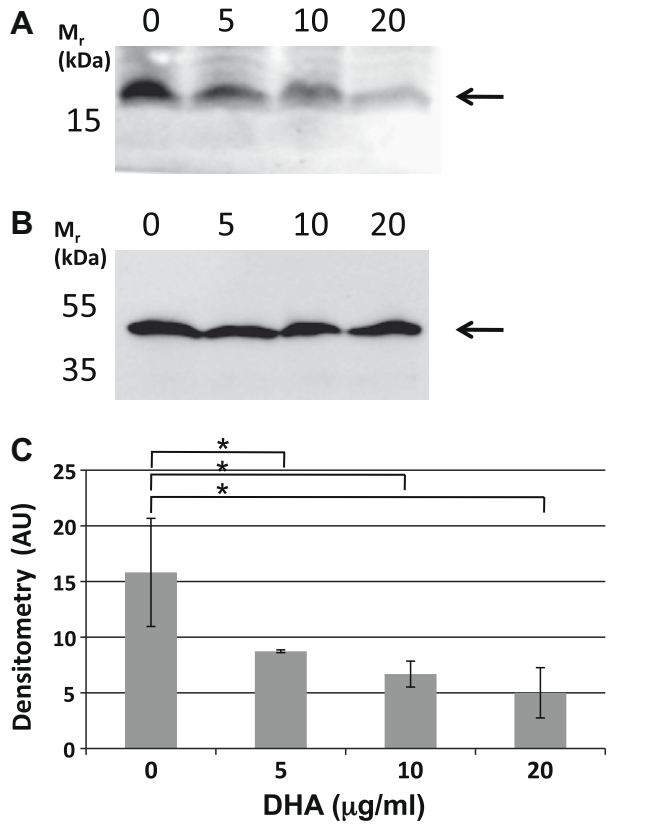

Fig. 2. Western blot analysis of apoptosis in M17 cells. Caspase-3 (A) and the housekeeping β-actin (B) protein levels (arrows) of M17 cells cultured in the absence and presence (5, 10 and 20 μg/ml) of DHA were detected with specific antibodies. (C) Densitometric analysis of caspase-3 protein levels (normalised with the β-actin protein levels) are shown in arbitrary units (AU). Molecular mass protein markers (M_r) are indicated on the left of each gel. This result is representative of three similar independent experiments. The data are shown as means ($n = 3$, $^*P < 0.05$).

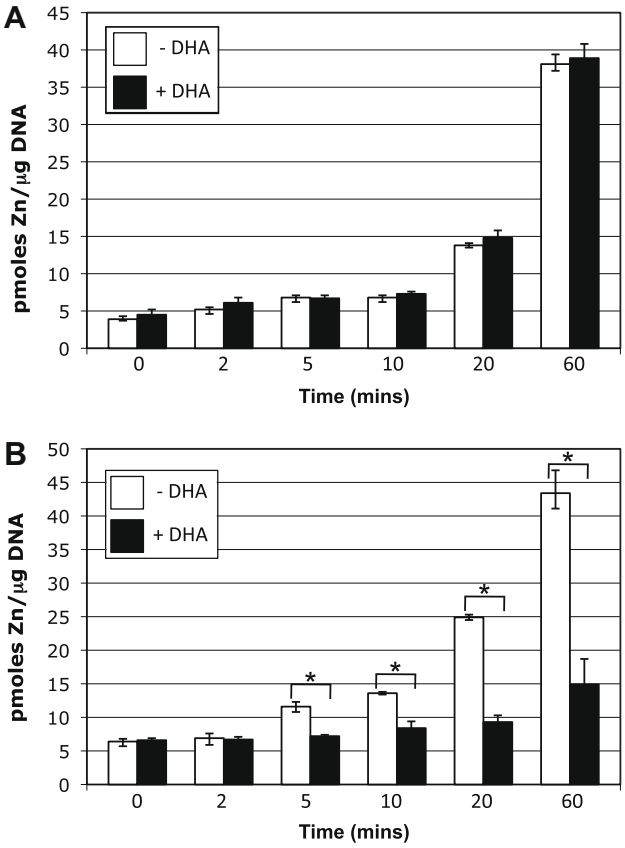

Fig. 3. Radiolabelled zinc influx studies. M17 cells were cultured either without (A) or with 20 μg/ml DHA for 2 days (B) prior to zinc influx studies using ^{65}Zn. Accumulation (influx) of Zn (pmol/μg DNA) in the cells was measured at different time points (min) before and after exposure to ^{65}Zn in the absence (empty bars) and presence (solid bars) of 20 μg/ml DHA. The data are shown as means ($n = 3$, $^*P < 0.05$).

absence of DHA over a period of 1 h. The zinc levels in M17 cell pellets were similar in the presence of 20 μg/ml DHA and in the control culture medium without DHA (Fig. 3A).

3.5. M17 cells that had incorporated DHA showed reduced cellular zinc influx

M17 cells were grown in culture medium enriched with DHA for 2 days prior to exposure to ^{65}Zn for a period of 1 h. DHA-treated cells showed reduced cellular zinc accumulation over a period of one hour (Fig. 3B). The decrease in zinc uptake was significant at 5 min and more pronounced at subsequent time intervals, relative to untreated cells. By 1 h, DHA-treated M17 cells had accumulated only 35% of the amount of zinc of untreated cells.

3.6. M17 cells express a range of zinc transporters belonging to the SLC30 and SLC39 families

Real-time PCR (Q-PCR) was used to measure the mRNA expression levels of members of the two major families of zinc transporters SLC30 and SLC39, in M17 cells, relative to expression of ZnT3 (Fig. 4). The M17 cells expressed all of the transporters tested. The most highly expressed zinc transporters were ZnT6 and ZnT7.

3.7. DHA reduced mRNA levels of ZnT3 in M17cells

Since ZnT3 has a potentially significant role in the brain as a putative transporter of zinc into synaptic vesicles, we next investigated the effect of DHA on ZnT3 expression levels. Real-time PCR analysis of M17 cells grown either in DHA-enriched or DHA-deficient culture medium indicated that ZnT3 mRNA expression levels were reduced by more than 12-fold in the presence of 40 μg/ml DHA (Fig. 5).

3.8. DHA reduced protein expression levels of ZnT3 in M17 cells

Western blot analysis to assess changes in ZnT3 protein levels in response to DHA treatment indicated that in cells exposed to 20 μg/ml DHA, ZnT3 protein levels were markedly reduced when compared with the untreated cells (Fig. 6A), and normalisation to the β-actin housekeeping gene (Fig. 6B) indicated a greater than 47% reduction in ZnT3 protein levels with 20 μg/ml DHA compared with the untreated cells (Fig. 6C).

3.9. DHA does not affect ZnT3 localisation in M17 cells

Immunolocalisation studies to assess whether DHA influenced the localisation of ZnT3 in M17 neuronal cells showed a similar granular cytoplasmic localisation both in M17 cells cultured with (Fig. 7A) and without (Fig. 7B) DHA. This is consistent with the intracellular vesicular localisation previously described for ZnT3.

4. Discussion

Both DHA and zinc play crucial, but seemingly independent roles in brain function. Alterations in both DHA levels and zinc

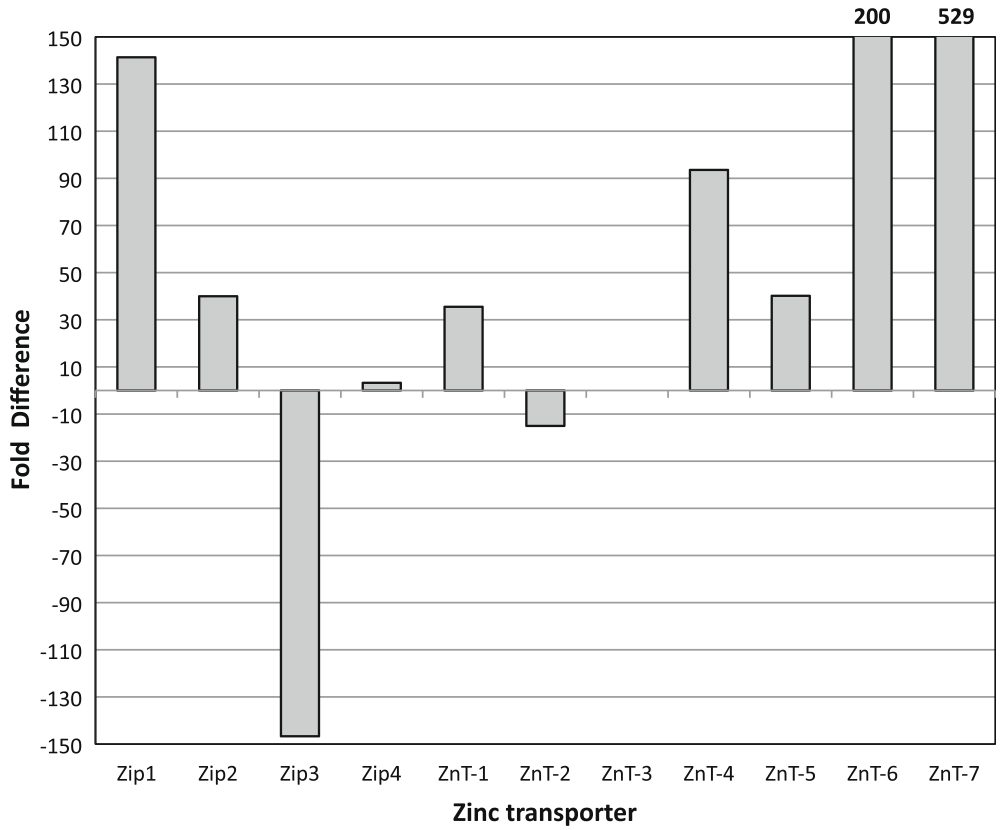

Fig. 4. Real-time PCR analysis of the different zinc transporters in M17 cell line. Fold-difference of mRNA levels of the different putative zinc influx (Zip1–Zip4) and efflux (ZnT1–ZnT7) transporters measured relative to that of ZnT3.

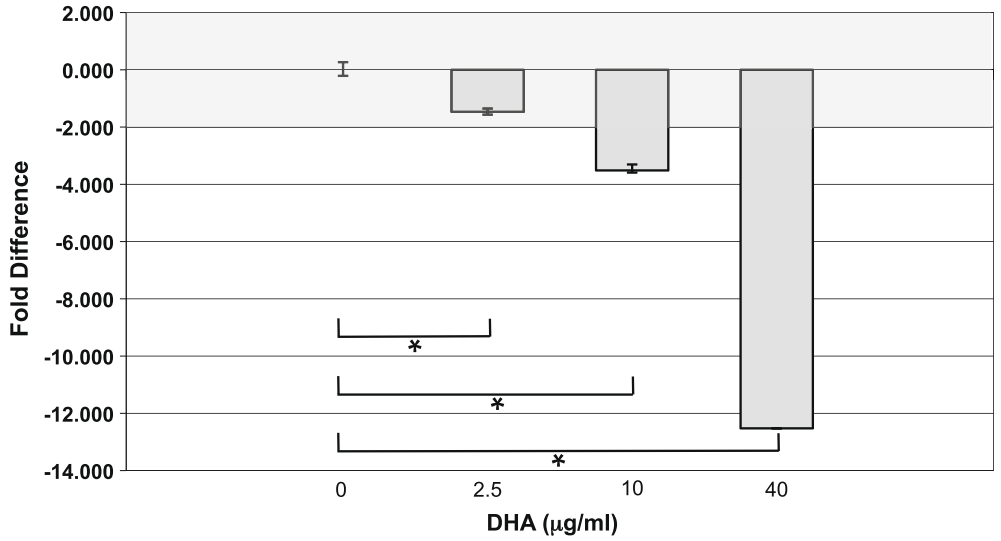

Fig. 5. Real-time PCR analysis of ZnT3 in M17 cells. M17 cells cultured in the absence and in the presence of different DHA concentrations (μg/ml) showing fold-difference of mRNA levels (solid bars) when compared to the no DHA control. Fold-difference of less than 2 and more than −2 (grey shaded area) can be considered as unreliable background noise and therefore not significant. The data are shown as means ($n = 3$, $^*P < 0.05$).

homeostasis are key features of degenerative brain disorders [8,26]. Our previous data revealed a link between DHA deficiency and altered zinc homeostasis in the brain of rats fed on a DHA-deficient diet [15]. Although these data demonstrated a clear relationship between DHA metabolism and zinc homeostasis, the molecular mechanisms of this interaction have not been elucidated. In the current study, we have shown that DHA reduces cellular zinc uptake possibly mediated by the zinc transporter ZnT3, with subsequent reduced apoptotic cell death. This indicates that the adverse effects of dietary DHA deficiency in promoting neurodegenerative brain disorders may be mediated, at least in part, through altered zinc fluxes.

We tested the hypothesis that the link between zinc and DHA may be due to a shared plasma membrane transport process such

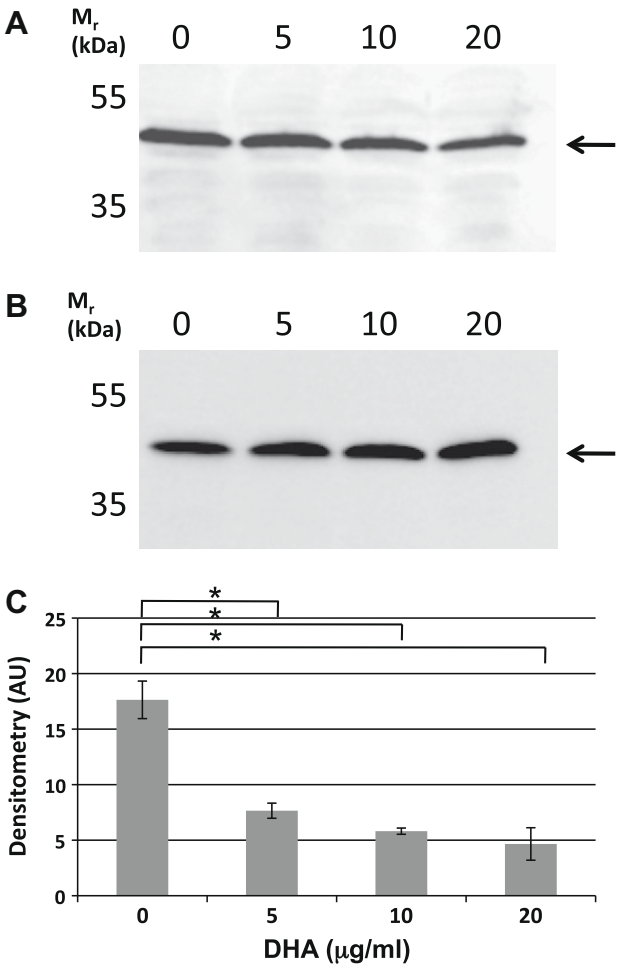

Fig. 6. Western blot analysis of ZnT3 expression in M17 cells. ZnT3 (A) and the housekeeping β-actin (B) protein levels (arrows) of M17 cells cultured in the absence and presence (5, 10 and 20 μg/ml) of DHA for 2 days were detected with specific antibodies. (C) Densitometric analysis of ZnT3 protein levels (normalised with the β-actin protein levels) are shown in arbitrary units (AU). Molecular mass protein markers (M_r) are indicated on the left of each gel. Data is representative of three similar independent experiments. The data are shown as means ($n = 3$, $^*P < 0.05$).

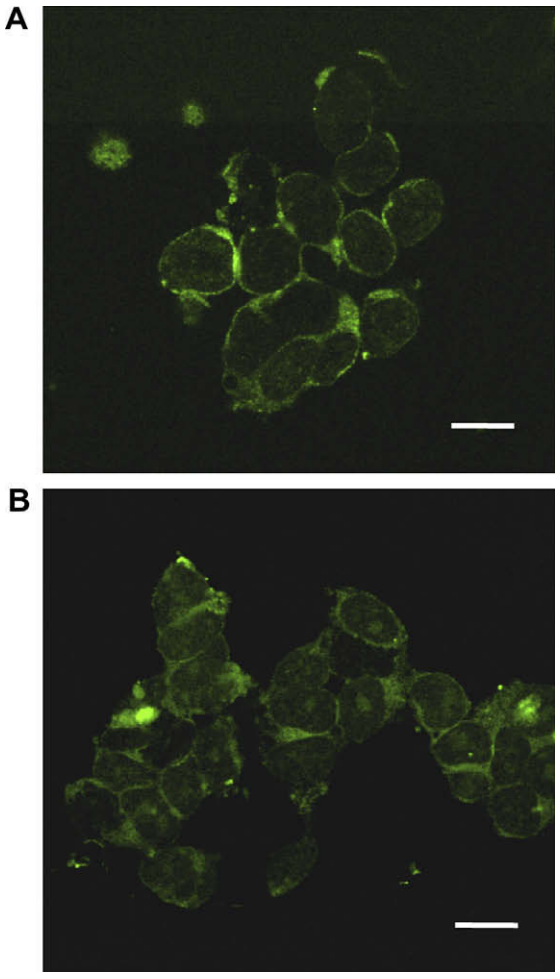

Fig. 7. Immunofluorescence analysis of ZnT3 protein localisation in M17 cells. Cells were cultured either with 20 μg/ml DHA (A) or without DHA (B) for 2 days and ZnT3 proteins detected with ZnT3-specific primary antibody, followed by fluorescent secondary antibodies. Scale bars represent 20 μm.

as a co-transport mechanism, as a link between zinc and PUFA metabolism is well established, where the clinical symptoms of zinc deficiency and PUFA deficiency are similar; disturbances in lipid metabolism occur in zinc deficient patients and zinc-deficient pregnant rats have impaired transport of PUFA to the foetus [16]. We excluded the presence of a zinc–PUFA membrane transport system. This was proven through data showing that DHA in the culture medium did not affect ^{65}Zn uptake over an 1 h period. Thus, the membrane transport processes for zinc and DHA do not appear to be linked. When M17 cells were grown in the presence of DHA for 2 days prior to the addition ^{65}Zn, however, a 65% reduction in zinc accumulation was seen over a period of 1 h, providing direct evidence that incorporated cellular DHA can influence zinc fluxes. Significantly, this cell culture data is consistent with our previous results that showed an increase in zinc levels in the synaptic vesicles of rodents on DHA-deficient diets [15].

Zinc homeostasis in the brain, including the uptake and efflux of zinc from cells, is regulated by membrane-bound zinc transporters belonging to members of the solute carrier 39 (SLC39) and the solute carrier 30 (SLC30), respectively [27,28]. The SLC39 transporters are involved in the uptake of zinc into the cytoplasm while zinc transporters of the SLC30 class, also known as ZnT, are involved in compartmentalization of zinc from the cytoplasm into intracellular vesicles or across the plasma membrane. In M17 cells, we detected expression of numerous members of these two families including Zip1, Zip2, Zip3, Zip4, ZnT1, ZnT2, ZnT3, ZnT4, ZnT5, ZnT6 and ZnT7. The relative expression levels of these genes varied considerably. A range of zinc transporters are expressed in the mouse brain, including ZnT1, ZnT3, ZnT4, ZnT5, ZnT6 and ZnT7 [29]. Our results using M17 cells confirm that human neuronal cells express many members of the ZnT family as well as the Zip family.

We focused our further studies on ZnT3 in relation to the effects of DHA as this transporter is associated with brain zinc accumulation as well as Alzheimer's disease. ZnT3 co-localises with zinc pools in the mouse brain [30] and *Slc30a3* knockout mice show reduced zinc in hippocampal mossy fibres [31]. ZnT3 is upregulated in Alzheimer's disease [29] and in APP/PS1 transgenic mice [32] and the APP/ΔZnT3 double knockout mouse does not develop Alzheimer's symptoms, indicating a critical role for ZnT3 in the pathogenesis of Alzheimer's disease [33].

We previously showed that ZnT3 mRNA transcripts were increased in the brains of rats on a DHA-deficient diet relative to control animals [15]. This relationship was also seen here where exposure of M17 cells to DHA concentrations of 10 and 40 μg/ml decreased the expression of ZnT3 by 3.5 and more than 12-fold, respectively, and was mirrored by a reduction in ZnT3 protein

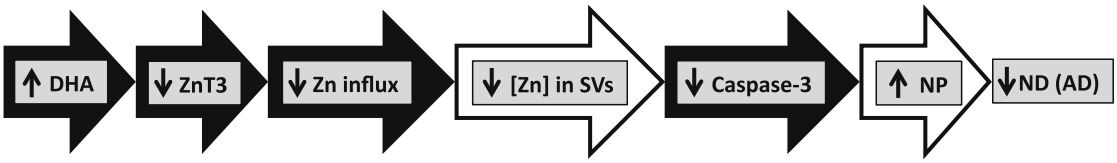

Fig. 8. Schematic diagram of the mechanism of DHA in M17 cells. The key findings of this study are represented by the solid (black) arrows. Empty (white) arrows represent possible benefits of DHA in neuroprotection (NP) and lowering of zinc concentrations ([Zn]) in neuronal synaptic vesicles (SVs) and therefore contributing to lower incidence of neurodegenerative diseases (ND), like Alzheimer's disease (AD).

levels with increasing DHA concentrations. Since ZnT3 was located in the cytoplasmic region of M17 cells, with a granular vesicular distribution, DHA may function to decrease the ZnT3-mediated transport of zinc from cytoplasm into intracellular vesicles. To maintain zinc homeostasis and constant cytoplasmic zinc levels, zinc uptake across the plasma membrane may therefore be reduced.

Degenerative death of neurons in cortical brain regions is a key feature of neurodegenerative disorders, including Alzheimer's disease. Multiple evidence from studies in experimental models and from brain tissue indicates that this neurodegeneration is a result of neuronal cell apoptosis [34]. We found a direct link between DHA treatment and inhibition of apoptosis in M17 cells where more than a 66% reduction in active caspase-3 protein levels was detected in cells treated with 20 μg/ml DHA, compared with the untreated cells. Investigations into the mechanism of apoptotic cell death indicate that the phosphatidylinositol 3-kinase/Akt signalling is a critical pathway in this process and DHA acts in this pathway. In cultured mouse neuroblastoma (Neuro 2A) cells, DHA increased phosophatidyl serine levels resulting in translocation and phosphorylation of Akt, dependent on phosphorylation of Akt at Thr-308, that suppressed activation of caspase-3 [10]. This Akt-dependent mechanism induced by DHA deficiency through phosphatidyl serine is not known to involve zinc. Zinc, however, phosphorylates Akt Thr-308 [35]. Zinc also directly activates Akt by phosphorylation at Ser-473/Thr-308 leading to activation of GSK-3beta and subsequent cell death, seen in cultured H19-7 embryonic hippocampal cells [36]. Thus, DHA may act to decrease cellular zinc levels leading to increased Akt activity and reduced apoptosis.

In summary, dietary DHA reduces the risk of cognitive decline that is associated with neurodegenerative conditions including Alzheimer's disease. There is strong evidence that DHA acts through inhibiting apoptosis. In the current study we have shown that the DHA-induced decrease of apoptosis in M17 cells is accompanied by a decrease in cellular zinc uptake and reduction of ZnT3 mRNA and protein levels. We propose that zinc has a key role in pathways that regulate brain cell survival and altered zinc homeostasis may contribute to the development of neurodegenerative disorders such as Alzheimer's disease. To illustrate this, a model of the DHA–zinc–apoptosis pathway is shown (Fig. 8, black arrows). Here, ZnT3 imports zinc into synaptic vesicles [30,31] to regulate zinc-dependent apoptosis. This model accounts for the positive effect of DHA in reducing neuronal cell death and neurodegenerative diseases such as Alzheimer's disease (Fig. 8, white arrows), whose incidence is reduced in populations with a high omega-3 fatty acid diet.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.12.013.

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