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DHA inhibits ER Ca²⁺ release and ER stress in astrocytes following *in vitro* ischemia

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Abstract

Docosahexaenoic acid (DHA) has neuroprotective effects in several neurodegenerative disease conditions. However, the underlying mechanisms are not well understood. In the present study, we investigated the effects of DHA on astrocyte Ca²⁺ signaling under *in vitro* ischemic conditions (oxygen/glucose deprivation and reoxygenation, OGD/REOX). OGD (2 hour) triggered a Ca²⁺_{ER} store overload (~ 1.9 fold). Ca²⁺ uptake by the Ca²⁺_{ER} stores was further augmented during REOX and Ca²⁺_{ER} was elevated by ~ 4.7-fold at 90 min REOX. Interestingly, Ca²⁺_{ER} stores abruptly released Ca²⁺ at ~ 120 min REOX and emptied at 160 min REOX. Depletion of Ca²⁺_{ER} stores led to delayed elevation of intracellular Ca²⁺ concentration (Ca²⁺_{cyt}) and cell death. Activation of the purinergic receptor P2Y1 was responsible for the release of Ca²⁺_{ER}. Most importantly, DHA blocked the initial Ca²⁺_{ER} store overload, the delayed depletion of Ca²⁺_{ER}, and rise in Ca²⁺_{cyt}, which was in part via inhibiting IP₃ receptors. The DHA metabolite DiHDoHE exhibited similar effects. DHA also attenuated expression of phosphorylated eukaryotic initiation factor 2α and activating transcription factor-4, two ER stress markers, following *in vitro* ischemia. Taken together, these findings suggest that DHA has protective effects in astrocytes following *in vitro* ischemia, in part, by inhibiting Ca²⁺ dysregulation and ER stress.

Keywords

ER Ca²⁺; IP₃ receptor; ryanodine receptor; polyunsaturated fatty acid; ER stress

INTRODUCTION

Docosahexaenoic acid (DHA, 22:6n-3) is the most abundant of the polyunsaturated fatty acids (PUFAs) in the brain (Kim 2007; Bazan 2006). DHA has been shown to promote neuronal survival and differentiation (Kim 2007; Bazan 2006). DHA or its oxidized products, such as neuroprotection D1 (NPD1, 10,17S-docosatriene), are neuroprotective in several neurodegenerative disease conditions, including experimental stroke, Alzheimer's disease, retinal degeneration, and spinal cord injury (Bazan 2006; Bazan 2007; King et al. 2006). Although the molecular mechanisms underlying the biological activities of DHA remain to be fully understood, NPD1 has been shown to inhibit apoptotic and inflammatory signaling in human neuronal cells against Aβ₄₂-induced cytotoxicity or in ischemic brains (Lukiw et al. 2005; Marcheselli et al. 2003; Belayev et al. 2005).

Astrocytes are the only brain cell type with the capacity to synthesize DHA (Moore et al. 1991). DHA is biosynthesized in astrocytes from α-linolenic acid through chain elongation and desaturation processes by Δ^{4–6} desaturases (Sprecher 2000). Neurons cannot produce DHA due to the lack of desaturase activity and thus depend on astrocytes to supply DHA (Moore et al. 1991). However, the role of DHA in astrocyte function has not been extensively studied. Many astrocyte functions are known to be regulated by changes of intracellular Ca²⁺ concentration (Ca²⁺_{cyt}) and Ca²⁺ release from the endoplasmic reticulum (ER) Ca²⁺ (Ca²⁺_{ER}) stores. DHA supplementation enhances gap junction coupling capacity in rat cortical astrocytes (Champeil-Potokar et al. 2006). Moreover, DHA suppresses thrombin-evoked Ca²⁺ response, inhibits store-operated Ca²⁺ entry (SOCE), and ATP-mediated intracellular Ca²⁺ oscillations in rat astrocytes (Sergeeva et al. 2002; Sergeeva et al. 2005). On the other hand, disturbance of Ca²⁺ homeostasis in the ER causes ER stress and activates the unfolded protein response (UPR) (Kaufman 1999). Changes in ER Ca²⁺ stores in astrocytes precede ER stress and mitochondrial dysfunction and are involved in

astrocyte damage (Liu et al. 2010). However, the effect of DHA on Ca^{2+} signaling and ER stress in astrocytes is unknown.

In the present study, we report that OGD/REOX triggered a biphasic change in ER Ca^{2+} stores (overload and depletion). DHA blocked the ER Ca^{2+} overload and depletion of $\text{Ca}^{2+}_{\text{ER}}$ stores, as well as, the subsequent delayed rise in $\text{Ca}^{2+}_{\text{cyt}}$ in astrocytes following *in vitro* ischemia. In addition, DHA attenuated ER stress and astrocyte death. Taken together, these findings suggest that the neuroprotective effects of DHA following ischemia may be, in part, through inhibiting astrocyte Ca^{2+} dysregulation and ER stress.

METHODS

Materials

Eagle's modified essential medium (EMEM) and Hanks balanced salt solution (HBSS) were from Mediatech Cellgro (Manassas, VA). Fetal bovine serum (FBS) was obtained from Valley Biomedical (Winchester, VA). Collagen-type I, 4-bromo-A23187, adenosine 5'-Triphosphate (ATP), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), 2-Methylthioadenosine- 5'- O- diphosphate (2Me-S-ADP), 6-Methyl-2-(phenylethynyl) pyridine hydrochloride (MPEP), and saponin were purchased from Sigma Chemicals (St. Louis, MO). D-myo-inositol 1,4,5-triphosphate (IP_3), mag fura-2 acetoxymethyl ester (Mag-fura 2-AM), and fura-2 acetoxymethyl ester (Fura 2-AM) were from Invitrogen (Carlsbad, CA). 2 ϵ -Deoxy-N6-methyladenosine 3 ϵ ,5 ϵ -bisphosphate (MRS 2179), 2-Aminoethoxydiphenylborane (2-APB), and ryanodine were from Tocris (Ellisville, MO). 2-phenyl-1,2-benziselenazol-d(2H)-one (Ebselen) and 10(S),17(S)-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid (DiHDoHE) were from Cayman Chemical (Ann Arbor, MI). Mouse anti α -tubulin monoclonal antibody and rabbit anti phosphor-eIF2 α monoclonal antibodies was purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-activating transcription factor 4 (ATF-4) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Primary culture of mouse cortical astrocytes

Dissociated cortical astrocyte cultures were established as described before (Su et al. 2002). Cerebral cortices were removed from 2–3 day-old mice. The cortices were incubated in a trypsin solution (0.25 mg/ml of HBSS) for 20 min at 37°C. The dissociated cells were rinsed and re-suspended in EMEM containing 10% FBS. Viable cells (1×10^4 cells/well) were plated in 6-well plates containing type 1 collagen coated glass cover slips (22 mm $\times$ 22 mm) or on 75 cm² flasks. Cultures were maintained in a 5% CO_2 atmosphere at 37°C. To obtain morphologically differentiated astrocytes, confluent cultures (7 days in culture, DIV 7) were treated with EMEM containing 0.25 mM dibutyryl cyclic AMP (dBcAMP) to induce differentiation. Experiments were routinely performed in DIV 10–15 cultures.

In vitro hypoxia/ischemia

Astrocytes on coverslips were rinsed twice with an isotonic OGD solution (pH 7.4) containing (in mM, at 37°C): 0 glucose, 21 NaHCO_3 , 120 NaCl , 5.36 KCl , 0.33 Na_2HPO_4 , 0.44 KH_2PO_4 , 1.27 CaCl_2 , 0.81 MgSO_4 (Kintner et al. 2007). Cells were incubated in 1.0 ml of the OGD solution in a hypoxic incubator for 2 h (Forma Scientific Inc., model 3130, Marietta, OH), containing 94% N_2 , 1% O_2 and 5% CO_2 . An orbital shaker (Thermolyne Inc, model M48215, Dubuque, IA) in the hypoxic chamber was used to facilitate equilibration of the hypoxic gases during the initial 30 min OGD. For REOX, OGD-treated cells were superfused with HEPES-MEM buffer containing 5.5 mM glucose for 0–180 min. Normoxic control cells were incubated in 5% CO_2 and atmospheric air in an isotonic control solution for 2 h.

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) measurement

Astrocytes were incubated with 5 μM fura-2 AM for 30–120 min at 37°C (Lenart et al. 2004). The cells were washed and the coverslips placed in the imaging chamber with HEPES-MEM at 37°C. Using a Nikon TiE 300 inverted epifluorescence microscope (40X oil immersion objective lens), astrocytes were excited every 10–30 seconds at 340 and 380 nm and the emission fluorescence at 510 nm was recorded. Images were collected and analyzed with the MetaFluor (Molecular Devices, Sunnyvale, CA) image-processing software. At the end of each experiment, the cells were exposed to 1 mM MnCl_2 in Ca^{2+} -free HEPES-MEM. The MnCl_2 -corrected 340/380 emission ratios were converted to concentration as described previously (Lenart et al. 2004).

ER Ca^{2+} ($[\text{Ca}^{2+}]_{\text{ER}}$) measurement

Astrocytes on coverslips were incubated with 4 μM mag-fura-2 AM and 0.02 % pluronic acid during the last 30 min of 2 h OGD. For REOX, the coverslip was quickly (< 2 min) placed on an open-bath imaging chamber in HEPES-MEM at 37°C. Cells were excited every 10 sec at 345 and 385 nm and the emission fluorescence images were collected at 510 nm using Nikon TiE 300 inverted epifluorescence microscope and a 40X objective lens. $\text{Ca}^{2+}_{\text{ER}}$ were determined as described previously (Chen et al. 2008). Briefly, the cytosolic mag-fura-2 signal was eliminated when the plasma membrane was permeabilized with a 30 sec exposure to saponin (3.0 $\mu\text{g}/\text{ml}$) (Chen et al. 2008). The $\text{Ca}^{2+}_{\text{ER}}$ values were then

calculated using the equation $[\text{Ca}^{2+}_{\text{ER}}] = K \times (R_{\text{base}} - R_{\text{min}}) / (R_{\text{max}} - R_{\text{base}})$. K was determined as 72 μM in astrocytes using solutions of known Ca^{2+} concentrations (Calcium Calibration Buffer Kit, Invitrogen, Carlsbad, CA). R_{min} was obtained with a minimum $F_{345/385}$ ratio in a Ca^{2+} -free solution. R_{max} was the maximum $F_{345/385}$ ratio in a high Ca^{2+} solution (10 mM CaCl_2).

Gel Electrophoresis and Western Blotting

Cells were washed with ice cold phosphate-buffered saline (PBS, pH 7.4) and lysed with 30 sec sonication at 4°C in anti-phosphatase buffer (pH 7.4) containing (mM): 145 NaCl, 1.8 NaH_2PO_4 , 8.6 Na_2HPO_4 , 100 NaF, 10 $\text{Na}_4\text{P}_2\text{O}_7$, 2 Na_3VO_4 , 2 EDTA and 0.2 μM microcystin and protease inhibitors as described previously (Luo et al. 2005). Protein content was determined by the bicinchoninic acid method. Lysate protein samples (30 μg) and pre-stained molecular mass markers (Bio-Rad, Hercules, CA) were resolved by SDS-PAGE (4–15% gradient gel or 10% gel). The resolved proteins were transferred onto a nitrocellulose membrane. The blots were blocked with 7.5% nonfat dry milk in Tris-buffered saline containing Tween 20 (0.1%) for 2 h at room temperature and then incubated with a primary antibody at 4°C overnight. After rinsing, the blots were incubated with horseradish peroxidase-conjugated secondary IgG for 1 h at room temperature. Bound antibody was visualized using an enhanced chemiluminescence assay (Amersham Corp, Piscataway, NJ). Relative changes in protein expression were estimated from the mean pixel density of each protein band using Image J software. The following antibodies and dilutions were used: rabbit anti-phospho-eIF2 α (p-eIF2 α , 1:1000, polyclonal), rabbit anti-ATF-4 (1:500, polyclonal) and mouse anti- α tubulin (1:8000, monoclonal).

STATISTICS

Statistical significance was determined by student's *t*-test or an ANOVA (Bonferroni post-hoc test) in the case of multiple comparisons. A *P*-value < 0.05 was considered statistically significant. *N* values represent the number of different cultures used in each experiment.

RESULTS

Effects of DHA on P2Y receptor-mediated Ca^{2+} transient in normoxic astrocytes

Astrocytes express abundant purinergic P2Y receptors (Fumagalli et al. 2003). When ATP (100 μM), the physiological ligand of P2Y receptors, was applied to astrocytes under extracellular Ca^{2+} -free conditions, there was an immediate transient increase in $\text{Ca}^{2+}_{\text{cyt}}$ with a peak value of 1048 ± 173 nM that resolved by 3–4 min (Figure 1 A). The mean integrated Ca^{2+} release ($\text{IA}_{\text{Ca}^{2+}}$) was 240 ± 50 nM*min (data not shown). A similar response occurred when cells were exposed to the specific P2Y1 agonist 2Me-S-ADP (10 μM , Figure 1 A). Activation of the P2Y1 receptor by 2Me-S-ADP triggered a similar increase in the Ca^{2+} transient ($\text{IA}_{\text{Ca}^{2+}}$, 187 ± 17 nM*min). This implies that the ATP-mediated response can be largely attributable to activation of the P2Y1 receptor. This was confirmed by the effects of the specific P2Y1 receptor antagonist MRS-2179. As shown in Figure 1 (A, B), MRS-2179 (20 μM) blocked ~ 80% of the 2Me-S-ADP -induced transient increase in $\text{Ca}^{2+}_{\text{cyt}}$ ($\text{IA}_{\text{Ca}^{2+}}$, 37 ± 12 nM*min, $p < 0.05$).

Interestingly, DHA affected the P2Y1-mediated rise in $\text{Ca}^{2+}_{\text{cyt}}$. As shown in Figure 1 C (inset), in the presence of 10 μM DHA, 2Me-S-ADP (10 μM) failed to trigger the $\text{Ca}^{2+}_{\text{cyt}}$ transient under extracellular Ca^{2+} -free conditions. $\text{IA}_{\text{Ca}^{2+}}$ following 2Me-S-ADP application was significantly suppressed in a dose-dependent manner (2.5, 5, and 10 μM DHA). 10 μM DHA blocked nearly ~90% of the P2Y1 receptor-mediated increase in $\text{Ca}^{2+}_{\text{cyt}}$ (Figure 1 C).

Effects of DHA on IP_3 -mediated ER Ca^{2+} release in normoxic astrocytes

Effect of DHA on IP_3 receptor function was further investigated in normoxic conditions. In these experiments, the basal level of $\text{Ca}^{2+}_{\text{ER}}$ in astrocytes was 28.1 ± 1.1 μM (Figure 2 A, B). Application of 10 μM IP_3 to these cells triggered an immediate release of $\text{Ca}^{2+}_{\text{ER}}$. $\text{Ca}^{2+}_{\text{ER}}$ decreased steadily to ~ 5 μM over 2–3 min in the presence of IP_3 (Figure 2 A, B). Astrocytes were treated with 10 μM DHA (prior to permeabilization) for 3 min. The basal level of $\text{Ca}^{2+}_{\text{ER}}$ was not affected by the DHA treatment (29.1 ± 0.6 μM). However, in the presence of DHA (10 μM), addition of IP_3 caused a much slower initial release of $\text{Ca}^{2+}_{\text{ER}}$ (9.5 ± 2.6 $\mu\text{M}/\text{min}$ vs. 39 ± 6 $\mu\text{M}/\text{min}$, $p < 0.05$). At steady state, $\text{Ca}^{2+}_{\text{ER}}$ release was only 37 % in the presence of IP_3 and DHA (10 μM), compared to ~ 80% release in controls (Figure 2 B). Increasing DHA to 20 μM nearly blocked the IP_3 -mediated $\text{Ca}^{2+}_{\text{ER}}$ release (23.5 ± 0.2 μM vs. control value of 28.7 ± 1.3 μM $p < 0.05$, Figure 2 A, B). These results clearly imply that DHA can directly inhibit IP_3 receptor-mediated $\text{Ca}^{2+}_{\text{ER}}$ release.

DHA does not affect store-operated Ca^{2+} entry following depletion of $\text{Ca}^{2+}_{\text{ER}}$ stores in normoxic astrocytes

It has been reported that DHA inhibits SOCE and prevents refilling of $\text{Ca}^{2+}_{\text{ER}}$ stores following their depletion in undifferentiated rat astrocytes (Sergeeva et al. 2002). In the current study, depletion of the Ca^{2+} ER stores was induced by G-protein coupled P2Y1 receptor activation. In order to test whether DHA affects SOCE in mouse cortical astrocytes, $\text{Ca}^{2+}_{\text{ER}}$ stores were emptied with P2Y1 specific agonist 2Me-S-ADP (10 μM) in the absence of extracellular Ca^{2+} . Following return of extracellular Ca^{2+} , an increase in $\text{Ca}^{2+}_{\text{cyt}}$ (a peak value of 47 ± 12 nM) was detected upon activation of SOCE (Figure 3 A, B). Adding DHA (2.5–10 μM) did not significantly affect SOCE in mouse astrocytes ($p > 0.05$). To further investigate the effects of DHA on SOCE, thapsigargin (2 μM) was used to deplete intracellular Ca^{2+} stores in the absence of extracellular Ca^{2+} . As shown in Figure 3C, $[\text{Ca}^{2+}]_{\text{cyt}}$ in astrocytes were elevated resulting from activation of SOCE after thapsigargin-induced $\text{Ca}^{2+}_{\text{ER}}$ release. In the presence of DHA (10 μM), activation of SOCE remained unchanged (a $\text{Ca}^{2+}_{\text{cyt}}$ peak value of 122.7 ± 10.6 nM vs. control value of 120.9 ± 13.1 nM).

These findings clearly suggest that DHA selectively modulates IP₃ receptor-mediated Ca²⁺_{ER} release but not the SOCE in mouse cortical astrocytes.

Effects of DHA on Ca²⁺_{cyt} and Ca²⁺_{ER} in astrocytes following *in vitro* ischemia

As shown in Figure 4 A, a low level of Ca²⁺_{cyt} (65.4 ± 7.2 nM) was detected during 3 h normoxic incubation. 2 h OGD did not trigger any significant changes of Ca²⁺_{cyt}. Furthermore, Ca²⁺_{cyt} remained at the basal level during ~ 100 min REOX. This coincided with the sustained ER Ca²⁺ uptake during 0–120 min REOX (Figure 4 B). Interestingly, Ca²⁺_{cyt} started rising by 120 min REOX and reached a plateau level of 353 ± 69 nM between 150 ~ 180 min REOX. In the presence of 10 μM DHA, elevation of Ca²⁺_{cyt} was largely suppressed (*p* < 0.05). The DHA-mediated effects may, in part, result from DHA-mediated blockage of Ca²⁺_{ER} release during REOX (see below).

We then investigated the possible effects of DHA on Ca²⁺_{ER} in ischemic astrocytes. As shown in Figure 4 B, basal [Ca²⁺]_{ER} was 27 ± 3 μM. It increased to 65 ± 4 μM at 2 h OGD (*p* < 0.05, Figure 4 B). [Ca²⁺]_{ER} was further augmented during 30–90 min REOX (*p* < 0.05) and reached a peak of 130 ± 3 μM (*p* < 0.05). However, an abrupt release of [Ca²⁺]_{ER} at a rate of 2 μM/min occurred beginning at ~ 100 min REOX. [Ca²⁺]_{ER} was reduced to basal levels by 140 min REOX (Figure 4 B). Interestingly, application of DHA (10 μM) at 0 min REOX prevented the overloading of Ca²⁺_{ER} and subsequent release at 120 min REOX (Figure 4 B).

We further investigated whether DHA itself or its oxidation products block changes of Ca²⁺_{ER}. We used the non-specific lipid oxygenase inhibitor ebselen to block the endogenous lipid oxygenase activity and prevent DHA oxidation (Walther et al. 1999). As shown in Figure 4 B, in the presence of ebselen (10 μM), DHA failed to block the loading of Ca²⁺_{ER} and the subsequent release following OGD/REOX. This suggests that the oxidized products of DHA via lipidoxygenase function were involved. Indeed, when we applied the DHA oxidation product DiHDoHE, an isoform of 10,17S-docosatriene (Chen et al. 2009; Serhan et al. 2006), OGD/REOX-induced changes in Ca²⁺_{ER} were largely inhibited, similar to DHA (Figure 4 B). Taken together, these results suggest that DHA oxidation products are important in maintaining astrocyte Ca²⁺_{ER} homeostasis under ischemic conditions.

Mechanisms underlying release of Ca²⁺_{ER} stores in astrocytes following *in vitro* ischemia

Ca²⁺ is released from ER primarily through the ryanodine receptor (RyR) and IP₃ receptor Ca²⁺ release channels (Henzi and MacDermott 1992). We examined whether the Ca²⁺_{ER} release results from IP₃R and/or RyR activation between 90 and 120 min REOX. As shown in Figure 4 C, inhibition of IP₃R with a non-selective inhibitor 2-APB (100 μM) reduced the OGD/REOX-mediated [Ca²⁺]_{ER} release by 60% (*p* < 0.05). These data imply that activation of IP₃R plays a partial role in the Ca²⁺_{ER} release. To assess the role of RyR in release of Ca²⁺_{ER} stores, we examined the effects of ryanodine on depletion of Ca²⁺_{ER} stores. When RyR was inhibited by 100 μM ryanodine, there was 34 % reduction of the OGD/REOX-mediated release of Ca²⁺_{ER} stores at 120 min REOX (*p* < 0.05). Moreover, in the presence of both 2-APB and ryanodine, the effects were additive and Ca²⁺_{ER} release was absent. In fact, [Ca²⁺]_{ER} was significantly higher than the peak values in the non-treated astrocytes (*p* < 0.05, data not shown). These findings suggest that both IP₃R and RyR are involved in Ca²⁺_{ER} release in astrocytes following *in vitro* ischemia.

To specifically investigate effects of DHA on ER Ca²⁺ efflux, cells were exposed to DHA (10 μM) at 80 min REOX, a time just prior to Ca²⁺_{ER} release. [Ca²⁺]_{ER} was subsequently monitored at 120, 140 or 160 min REOX. As shown in Figure 4 D, the delayed addition of DHA at 80 min REOX effectively blocked ER Ca²⁺ efflux. Similarly, blocking P2Y1

receptor activity with MRS2179 at 80 min REOX also prevented the ER Ca^{2+} efflux (Figure 4 D). These findings further suggest that DHA indeed prevents ER Ca^{2+} efflux in astrocytes under ischemic conditions, which involves IP_3 mediated release pathways.

Effect of P2Y receptor activation in astrocytes under ischemic conditions

Figure 5 A shows that P2Y1 receptor agonist 2Me-S-ADP triggered a transient $\text{Ca}^{2+}_{\text{cyt}}$ response in normoxic astrocytes. However, P2Y1 receptor activity was transiently inhibited following 2 h OGD (~ 60%) and partially recovered at 120 min REOX (Figure 5 B). These data suggest that P2Y1 receptor activation may contribute to delayed $\text{Ca}^{2+}_{\text{ER}}$ release and $\text{Ca}^{2+}_{\text{cyt}}$ rise. This speculation was supported by the finding that inhibition of P2Y1 receptor activity with MRS2179 significantly attenuated $\text{Ca}^{2+}_{\text{cyt}}$ dysregulation during 120 min REOX (Figure 5 C). In addition, inhibition of mGluR5 receptor by MPEP did not further inhibit the delayed $\text{Ca}^{2+}_{\text{cyt}}$ rise in mouse astrocytes in the presence of MRS2179 (Figure 5 D). These results suggest that REOX triggered $\text{Ca}^{2+}_{\text{cyt}}$ increase is largely mediated by the activation of P2Y1 receptors coupled to IP_3 mediated $\text{Ca}^{2+}_{\text{ER}}$ release.

Effect of DHA on astrocyte ER stress

Disruption in ER Ca^{2+} homeostasis can lead to unfolded protein response and development of ER stress. In our previous study, we have shown that disruption in ER Ca^{2+} homeostasis increases the expression of phosphorylated eIF2 α (p-eIF2 α) and ER stress following OGD/REOX (Liu et al. 2010). In the present study, we first investigated effects of DHA on expression of p-eIF2 α . As shown in Figure 6 A, expression of p-eIF2 α was increased during 1–3 h REOX (~ 3.4 folds). Treatment of astrocytes with DHA during 0–3 h REOX significantly attenuated p-eIF2 α protein expression ($p < 0.05$). Moreover, 1–3 h REOX led to significant elevation of ATF-4 protein in astrocytes (Figure 6 B). DHA also blocked the up-regulation of ATF-4 protein. These results collectively indicate that DHA not only blocks dysregulation of $\text{Ca}^{2+}_{\text{ER}}$ but also attenuates ER stress in astrocytes following OGD/REOX.

We further investigated whether suppression of ER stress via DHA would have an impact on cell survival. We calculated cell survival by accessing the fura-2 dye retention in astrocytes during 180 min REOX. A sudden loss of the fluorescent dye reflects loss of plasma membrane integrity and cell death (Kintner et al. 2007). As shown in Figure 6 C, under normoxic conditions, most astrocytes retained the fluorescence dye during 3 h of perfusion and exhibited little cell damage (< 5%). 2 h OGD and 3 h REOX reduced cell survival to $73 \pm 3\%$ (Figure 6 C, D). Cells died between 140 and 180 min of REOX (shown as an abrupt loss of the dye, Figure 6 C), which coincided with the increases in cytosolic Ca^{2+} and loss of ER Ca^{2+} (see Figure 4). Interestingly, when 10 μM DHA was present throughout REOX, cell survival was maintained at $92 \pm 2\%$ ($p < 0.05$, Figure 6 C). Taken together, these data suggest that DHA suppressed ER stress as well as astrocyte death following *in vitro* ischemia.

DISCUSSION

DHA attenuates P2Y1 receptor-mediated $\text{Ca}^{2+}_{\text{cyt}}$ transients

Astrocytes play a key role in the synthesis and supply of DHA within the brain (Moore 2001). DHA is released readily from the astroglial membranes under basal and stimulated conditions, and supplied to the neurons (Moore 2001). However, the effects of DHA on astrocyte function are not well studied. The functions of astrocytes are regulated by changes of intracellular Ca^{2+} concentration and much of the Ca^{2+} signal may come from the ER Ca^{2+} stores (Takano et al. 2009). DHA plays an important role in the regulation of intracellular Ca^{2+} in astrocytes. In rat astrocytes, DHA inhibits the SOCE, reduces the amplitude of Ca^{2+}

response to agonists of G protein coupled receptors, and suppresses intracellular Ca^{2+} oscillations (Sergeeva et al. 2005).

Interestingly, in our current study, we found that the P2Y1 receptor-mediated $\text{Ca}^{2+}_{\text{cyt}}$ increase was blocked by DHA in a dose-dependent manner. 10 μM DHA blocked nearly 90% of the P2Y1 receptor-mediated $\text{Ca}^{2+}_{\text{cyt}}$ increase. It is known that activation of P2Y1 receptors leads to phospholipase C activation and IP_3 generation, which triggers Ca^{2+} release from ER stores in astrocytes (James and Butt 2002). Our data show that DHA decreased the exogenous IP_3 -mediated $\text{Ca}^{2+}_{\text{ER}}$ release rate and reduced the amount of $\text{Ca}^{2+}_{\text{ER}}$ release by $\sim 40\%$. These data support our speculation that DHA can directly inhibit IP_3 receptor-mediated $\text{Ca}^{2+}_{\text{ER}}$ release.

In contrast to the finding of Sergeeva et al. that DHA inhibits SOCE in rat astrocytes (Sergeeva et al. 2005), in our study, DHA failed to inhibit SOCE following depletion of $\text{Ca}^{2+}_{\text{ER}}$ stores in mouse cortical astrocytes. Our findings suggest that DHA selectively modulates IP_3 receptor-mediated $\text{Ca}^{2+}_{\text{ER}}$ release but not the SOCE in mouse astrocytes.

Effects of DHA in regulating ER Ca^{2+} and ER stress

DHA is the most abundant essential omega-3 fatty acid in the brain and has been shown to be neuroprotective in numerous *in vivo* and *in vitro* experiments. For instance, DHA protects neuronal cells against serum starvation-mediated apoptosis by up-regulation of Akt and extracellular-signal-regulated kinase (ERK) (Akbar et al. 2005). Administration of DHA to rats stimulated the ERK and Bcl-2 mediated anti-apoptotic cascade in cerebral ischemia (Akbar et al. 2005; Pan et al. 2009). DHA treatment also suppresses lipopolysaccharide/interferon- γ stimulated release of interleukin-6 in cultured cortical glial cells (Pan et al. 2009). However, the function of DHA in regulation of astrocyte Ca^{2+} homeostasis under ischemic conditions is unknown.

We found that DHA blocked the ER Ca^{2+} release in ischemic astrocytes. It is well documented that IP_3 - and caffeine/ryanodine-sensitive stores are the two major ER Ca^{2+} stores in astrocytes (Golovina and Blaustein 2000). Activation of these two receptors and increases in $\text{Ca}^{2+}_{\text{cyt}}$ have been detected in astrocytes *in vivo* under ischemic conditions (Ding et al. 2009). We show in this study that inhibition of IP_3R is more effective in attenuating $\text{Ca}^{2+}_{\text{ER}}$ store release than RyR inhibition following OGD/REOX. Blocking both IP_3R and RyR had an additive effect and completely prevented $\text{Ca}^{2+}_{\text{ER}}$ release. These results suggest that both IP_3R and RyR are involved in $\text{Ca}^{2+}_{\text{ER}}$ release in astrocytes following *in vitro* ischemia. We concluded that DHA can attenuate $\text{Ca}^{2+}_{\text{ER}}$ release via inhibiting IP_3R . In addition to affecting $\text{Ca}^{2+}_{\text{ER}}$ release, exposing DHA to astrocytes also attenuated $\text{Ca}^{2+}_{\text{ER}}$ overload following OGD. We speculate that this, in part, results from DHA-mediated inhibition of ER Ca^{2+} -ATPase. This view is supported by a study that cardiac sarcoplasmic reticulum Ca^{2+} -ATPase activity was reduced by $\sim 30\%$ in rats fed with a DHA enriched diet (Taffet et al. 1993). The underlying mechanisms could involve changes of the composition and/or structure of the phospholipid membranes of ER, which can affect Ca^{2+} -ATPase protein turnover and activity (Taffet et al. 1993).

ER is a multifunctional organelle involved in folding and processing of proteins, intracellular Ca^{2+} homeostasis, and cell death signal activation (Baumann and Walz 2001). Disturbance of Ca^{2+} homeostasis and accumulation of unfolded proteins in the ER cause ER stress and activates UPR (Kaufman 1999). UPR results in the phosphorylation and activation of PERK (PKR-like ER kinase), which triggers a cascade of events including the phosphorylation of eIF2 α , inhibition of protein synthesis, and activation of apoptotic signals (Harding et al. 2002; DeGracia and Hu 2007; Paschen 2003). We detected a rapid up regulation of p-eIF2 α and ATF-4 proteins during 1–3 h REOX, which coincided with the ER

Ca²⁺ overload and subsequent depletion. Interestingly, DHA not only blocked the ER Ca²⁺ overload and release, it also reduced p-eIF2 α and ATF-4 expression and cell death. Taken together, our data strongly suggest that DHA may increase cellular tolerance to stress by prevention of Ca²⁺_{ER} overload, depletion of Ca²⁺ stores, and ER stress.

Contribution of bioactive DHA metabolites

DHA's effects on ER Ca²⁺ dysregulation can result from its direct action or through its metabolites. DHA can be transformed by a 15-lipoxygenases-like enzyme to NPD1 (Lukiw et al. 2005). In the presence of the 5 and 15 LOXs potent inhibitor ebselen, the effect of DHA on ER Ca²⁺ loading and depletion was blocked. These results clearly indicated involvement of DHA metabolites. A possible candidate is NPD1. This speculation was confirmed by the use of DiHDoHE, an isomer of NPD1. DiHDoHE blocked ER Ca²⁺ release in ischemic astrocytes, mimicking the DHA effects. NPD1 is neuroprotective in models of experimental stroke by down-regulating brain ischemia reperfusion-induced leukocyte infiltration, proinflammatory signaling, and infarct size (Bazan, NG et al., 2009). Our study suggests that NPD1 could reduce ischemic brain damage via decreasing astrocyte ER Ca²⁺ dysregulation and ER stress.

In summary, we report here that DHA and its oxidized product NPD1 blocked purinergic receptor P2Y1-mediated Ca²⁺ release from ER Ca²⁺ stores in part via directly inhibiting IP₃R. DHA also attenuated the delayed rise in Ca²⁺_{cyt}, ER stress, and astrocyte death following *in vitro* ischemia. Taken together, our findings suggest that the DHA-mediated protective effects in astrocytes may in part be attributable to its inhibition of astrocyte Ca²⁺ dysregulation and ER stress following *in vitro* ischemia.

Abbreviations

dBcAMP	dibutyryl cyclic AMP
[Ca²⁺]_{cyt}	calcium concentration cytoplasmic
[Ca²⁺]_{ER}	calcium concentration endoplasmic reticulum
DIV	days in culture
ER	endoplasmatic reticulum
FBS	fetal bovine serum
Fura-2 AM	Fura-2 acetoxymethyl ester
HBSS	Hanks balanced salt solution
IP₃R	inositol 1,4,5-triphosphate receptor
mGluR5	Group I metabotropic glutamate receptor 5
OGD/REOX	oxygen glucose deprivation and reoxygenation
p-eIF2α	phosphorylated eukaryotic initiation factor 2 alpha
RyR₂	ryanodine receptor type 2
DHA	Docosahexaenoic acid (22:6n-3)
2Me-S-ADP	methylthioadenosine- 5'- O- diphosphate
MPEP	6-Methyl-2-(phenylethynyl) pyridine hydrochloride
IP₃	D-myo-inositol 1,4,5-triphosphate
Mag-fura 2-AM	mag fura-2 acetoxymethyl ester

Fura 2-AM	fura-2 acetoxymethyl ester
2-APB	2-Aminoethoxydiphenylborane
Ebselen	2-phenyl-1,2-benzisoselenazol-d(2H)-one
DiHDoHE	10(S),17(S)-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid 10(S),17(S)-DiHDoHE
ATF-4	activating transcription factor 4
UPR	unfolded protein response
SOCE	store-operated Ca^{2+} entry

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Figure 1. DHA suppresses P2Y1 receptor-mediated Ca²⁺ transients in normoxic astrocytes
A. Representative tracing of changes in [Ca²⁺]_{cyt} induced by ATP (100 μM) and P2Y1 agonist 2Me-S-ADP (10 μM) under Ca²⁺ free conditions. Effects of P2Y1 antagonist MRS-2179 (20 μM) on 2MeSADP-mediated Ca²⁺ changes were tested. Data are means ± SEM, n = 3. **B.** Summary data. Integrated area (IA) under the Ca²⁺_{cyt} transient in response to 2Me-S-ADP was determined. Data are means ± SEM, n = 3. * p < 0.05 vs. 2Me-S-ADP. **C.** Effects of DHA (10 μM) on 2Me-S-ADP -induced Ca²⁺_{cyt} transient. Inset: Representative tracings showing pretreatment (3 min) of astrocytes with DHA (10 μM) prior to application of 2Me-S-ADP (10 μM) blocked the Ca²⁺_{cyt} transient. Data are means ± SEM, n = 3. * p < 0.05 vs. 2Me-S-ADP

Figure 2. Effects of DHA on IP₃-mediated changes in [Ca²⁺]_{ER} in normoxic astrocytes
A. Representative tracings of Ca²⁺_{ER} release in response to IP₃. IP₃ (10 μ M) was applied to saponin-permeabilized astrocytes to trigger the Ca²⁺_{ER} release. DHA (10 or 20 μ M) was added 2–3 min prior to permeabilization and present in subsequent solutions. **B.** Summary data. Data are means $\pm$ SEM, n = 3. * p < 0.001 vs. corresponding baseline, # p < 0.001 vs. Con IP₃.

Figure 3. DHA does not affect store-operated Ca^{2+} entry in astrocytes

A. Representative tracing showing release of Ca^{2+}_{ER} stores and store-operated Ca^{2+} entry (SOCE) in astrocytes. SOCE was determined after Ca^{2+}_{ER} stores were emptied by application of 10 μ M 2-MeSADP in the absence of extracellular Ca^{2+} (arrow). SOCE-mediated increase in $[Ca^{2+}]_{cyt}$ was detected when Ca^{2+} was reintroduced at 3–4 min. Inset: effects of DHA (10 μ M) on SOCE were tested following 2-MeSADP application, added 3–4 min prior to reintroduction of Ca^{2+} . **B.** Summary data of experiments in A. **C.** Representative traces showing thapsigargin-mediated release of Ca^{2+}_{ER} stores and activation of SOCE in astrocytes. SOCE was determined after Ca^{2+}_{ER} stores were emptied by

application of 2 μ M thapsigargin in the absence of extracellular Ca^{2+} . DHA (10 μ M) failed to block SOCE. Data are means $\pm$ SEM, n = 3–4.

Figure 4. Effect of DHA on changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{ER}$ in astrocytes following OGD and REOX

A. Changes of $[Ca^{2+}]_{cyt}$ in astrocytes was determined with fura-2. Cells were either exposed to 3 h normoxia or 2 h OGD and 0–180 min REOX. In the DHA (10 μ M) studies, DHA was present during REOX. Data are mean $\pm$ SEM, $n = 3$. * $p < 0.05$ vs. Normoxia control **B.** Changes of $[Ca^{2+}]_{ER}$ in astrocytes were determined with mag-fura-2. In the DHA (10 μ M), ebselen (10 μ M) or DiHDoHE (69 μ M) studies, the drugs were present during 0–180 min REOX. Data are mean $\pm$ SEM, $n = 3$ –7. * $p < 0.05$ vs. control, # $p < 0.05$ vs. OGD/REOX. **C.** Role of IP₃R and RyR in Ca^{2+}_{ER} release in astrocytes following OGD/REOX. Changes of $[Ca^{2+}]_{ER}$ were determined at 90 and 120 min REOX following 2 h OGD. For the drug treatment, either 2-APB (100 μ M), ryanodine (100 μ M), or 2-APB (100 μ M.) plus ryanodine (100 μ M) were added at 0 min REOX. Data are mean $\pm$ SEM, $n = 4$ –7. # $p < 0.05$ vs. OGD/REOX. **D.** Addition of DHA (10 μ M) or the P2Y1 antagonist MRS-2179 (20 μ M) at 80 min REOX prevents astrocytes from release of $[Ca^{2+}]_{ER}$ at 120, 140 or 160 min REOX. Data are mean $\pm$ SEM, $n = 3$. * $p < 0.05$ vs. corresponding REOX control.

Figure 5. P2Y1 receptor-mediated Ca^{2+} transients after OGD/REOX

A. 2Me-S-ADP (10 μ M)-induced Ca^{2+}_{cyt} transient in normoxic or OGD/REOX-treated cells. **B.** Summary data of IA under the Ca^{2+}_{cyt} transient in response to 2Me-S-ADP. Data are mean $\pm$ SEM, n = 3. * p < 0.05 vs. Normoxia, # p < 0.05 vs. OGD. **C.** Changes of $[Ca^{2+}]_{cyt}$ in astrocytes was determined with fura-2 under normoxic conditions and with OGD/REOX. In some studies, the P2Y1 antagonist MRS2179 (20 μ M) was present during 0–180 min h REOX. Data are mean $\pm$ SEM, n = 3. * p < 0.05 vs. normoxia, # p < 0.05 vs. OGD/REOX. **D.** The mGluR5 antagonist MPEP (50 μ M) did not provide further inhibition of OGD/REOX mediated rise in $[Ca^{2+}]_{cyt}$. Data are means $\pm$ SEM, n = 3. * p < 0.05 vs. normoxia, # p < 0.05 vs. OGD/REOX

Figure 6. DHA inhibits the OGD/REOX-mediated up regulation of ER stress proteins and cell death

A. Representative immunoblot showing regulation of p-eIF2 α protein in normoxic control, 2 h OGD, 1–3 h REOX. Data are mean $\pm$ SEM. $n=4$. * $p < 0.05$ vs. Con; # $p < 0.05$ vs. corresponding 1–3 h REOX. **B.** Representative immunoblot of ATF4 protein. DHA (10 μ M) was added during 1–3 h REOX. The same blot was probed with anti α -tubulin antibody as a loading control. Data are mean $\pm$ SEM. $n=4$. * $p < 0.05$ vs. Con; # $p < 0.05$ vs. corresponding 1–3 h REOX. **C.** Representative tracings of fura-2 fluorescence (Ca^{2+} -insensitive wavelength, 340 nm) during REOX following 2 h of OGD from 10 cells. The abrupt loss of fluorescence (arrow) indicates compromise of plasma membrane integrity and

cell death. **D.** Summary data of cell survival under normoxic conditions and at the end of 180 min REOX in OGD and OGD + DHA astrocytes. Data are mean $\pm$ SEM, n=3–4. * $p < 0.05$ vs. Con; # $p < 0.05$ vs. OGD/REOX.