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Targeting the Brain with a Neuroprotective Omega-3 Fatty Acid to Enhance Neurogenesis in Hypoxic Condition in Culture

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Abstract

Docosahexaenoic acid (DHA, 22:6n-3) is an essential omega-3 polyunsaturated fatty acid (PUFA) that is required for proper brain development and cerebral functions. While DHA deficiency in the brain was shown to be linked to the emergence of cerebral diseases, a dietary intake of omega-3 PUFA could prevent or attenuate neurologic disturbances linked with aging or neurodegenerative diseases. In this context, targeting the brain with DHA might offer great promise in developing new therapeutics for neurodegenerative diseases. We previously synthesized a stabilized form of DHA-containing lysophosphatidylcholine a major vector of DHA transportation to the brain, which is 1-acetyl,2-docoshexaenoyl-glycerophosphocholine, named AceDoPC®. Injection of AceDoPC® or DHA after experimental ischemic stroke showed that both molecules had neuroprotective effects but AceDoPC® was the most potent. This study aims to investigate the beneficial effects of DHA either unesterified or esterified within AceDoPC® on a model of neurogenesis in vitro, under physiological or pathological conditions. The effect of protectin DX (PDX, a double lipoxygenase product of DHA) was also tested. We cultured neural stem progenitor cells (NSPCs) derived from the adult mouse brain under normal or hypoxigenic (ischemic) conditions in vitro. Neurogenesis study of cell cultures with AceDoPC® showed enhanced neurogenesis compared to addition of unesterified DHA, PDX, or vehicle control, especially under pathological conditions. Our studies of the potential mechanisms involved in neuroprotection hinted that AceDoPC® neuroprotective and regenerative effects might be due in part to its anti-oxidative effects. These results indicate the potential for novel therapeutics against stroke that target the brain.

Keywords Docosahexaenoic acid · 1-acetyl,2-docoshexaenoyl-glycerophosphocholine · Protectin · Neuroprotection · Neurogenesis · Stroke · Brain

Introduction

Docosahexaenoic acid (DHA, 22:6n-3) is an omega-3 (ω-3) polyunsaturated fatty acid (PUFA) essential for human brain development, function, and visual acuity. It is specifically enriched in the brain and the retina. PUFA was shown to exhibit many health benefits in a great variety of diseases such as cardiovascular or neurodegenerative diseases [1–4]. Recent studies showed that dietary intake of omega-3 PUFA could prevent cerebral physiological disturbances due to aging or neurodegenerative diseases [5–10]. Deficiency in DHA level in the brain was shown to be linked with the emergence of neurodegenerative diseases such as Alzheimer's or Parkinson's disease [11].

DHA is only scarcely synthesized de novo in our metabolism [12] and is therefore mainly incorporated into our metabolism through dietary intake. Several groups evidenced that lysophosphatidylcholine-DHA (lysoPC DHA) is a major form of physiological transport of DHA through the blood-brain-barrier (BBB) [13–18]. The preference for 1-lyso,2-docosahexaenoyl-glycerophosphocholine was not observed with other organs such as heart, liver, and kidney [13]. As

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DHA migrates easily from the *sn*-2 position of lysoPC to the *sn*-1 position, we synthesized a molecule called 1-acetyl,2-docosahexaenoyl-glycerophosphocholine (AceDoPC®), which prevents the migration of DHA and retains physico-chemical properties close to the physiological carrier [19, 20].

DHA effects on brain functions and brain neuroprotection were covered by numerous studies. Notably, unesterified DHA could lessen the lesion size due to the induction of focal cerebral ischemia in vivo [4]. The decrease in lesion size was more important when DHA was esterified within AceDoPC® rather than unesterified. An in vitro model of cerebral ischemia named oxygen/glucose deprivation (OGD) was first established by Goldberg and Choi in 1993 [21]. Comparable neuroprotection of DHA was shown in neuronal cell cultures subjected to OGD [22]. Potential neuroprotective mechanisms include anti-oxidative, anti-inflammatory, and anti-apoptotic properties of DHA (for review, see Lo Van et al. 2016 [23]). DHA neuroprotective effect is expected to be due in part to its conversion into active oxygenated derived mediators, such as protectin D1 (PD1, called neuroprotectin D1 in brain), resolvins, and maresins [24, 25]. One isomer of PD1 was identified by our group and named protectin DX (PDX, Chen et al. [26]). Diverse neuroprotective effects of PD1 were reported in numerous reviews [27–29]. The stereo and geometric isomer PDX also exhibits anti-inflammatory, anti-aggregatory, and neuroprotective properties [30–33]. Other mechanisms behind the action of DHA includes modification of the membrane fluidity by modification of the fatty acid pattern of membrane phospholipids [34–38]. Study highlighted the beneficial effect of DHA on neurogenesis of embryonic neural stem/progenitor cells (NSPCs) [39]. In adult mammalian brains, neurogenesis mainly occurs in the subventricular zone of the lateral ventricles and in the subgranular zone of the dentate gyrus [40]. Adult neurogenesis may play a role in regeneration of diseased brain as well as in learning and memory [41]. These results are of particular interest since one therapeutic approach to cerebral diseases is to promote neurogenesis to counteract neuronal loss. Such neurogenesis-promoting effects by DHA were also shown in in vivo models, i.e., in the hippocampus of 18-month-old rats, respectively [42]. Moreover, experiments on transgenic fat-1 mice, which are able to convert omega-3 fatty acids from omega-6 fatty acids and thus having elevated levels of omega-3 fatty acids in their tissues, showed that high brain DHA levels increase neurogenesis and improve spatial learning [43].

This paper presents the effects of DHA, AceDoPC®, and PDX on NSPCs derived from the adult mouse brain as a model of adult neurogenesis in vitro. To mimic conditions of stroke in vitro, we cultured adult NSPCs under both physiological and pathological conditions, using OGD as a model of ischemia in vitro. We showed that AceDoPC® enhanced neurogenesis of adult NSPCs, especially when the cells were subjected to pathological conditions. The effect of AceDoPC® on neurogenesis was even higher than the effect of unesterified DHA.

Materials and Methods

Animals

All mice were either maintained at Tohoku University School of Medicine (Japan) or at INSA-Lyon (France) in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and with the European Communities Council Guidelines for the Care and Use of Laboratory Animals (22 September 2010, 2010/63/EU) in conformity with the Public Health Service (PHS) Policy on Human Care. All animal experiments were approved by the Tohoku University's committee for animal experiments (MED#2013-114) and carried out in compliance with French Ministry of Agriculture guidelines (no. 87–848). NSPCs were obtained from brains of C57BL/6 wild-type (WT) mice (Clea Japan, Tokyo, Japan; Envigo, Gannat, France).

Neural Stem/Progenitor Cell Extraction and Maintenance

NSPCs were obtained by primary culture with a protocol described previously with some modifications [44]. Wild-type mice (aged 8 to 10 weeks old) were sacrificed by cervical dislocation and were decapitated. The brain was removed and the subventricular zone of wild-type mouse brain were dissected and collected into an artificial cerebral spinal fluid containing: pure water; NaCl (0.12 M, Sigma-Aldrich, St. Louis, MO, USA); KCl (5 mM, Sigma-Aldrich); $MgCl_2$ (3 mM, Sigma-Aldrich); $CaCl_2$ (1 mM, Sigma-Aldrich); $NaHCO_3$ (2.19 mg/mL, Sigma-Aldrich); D-glucose (1.79 mg/mL, Sigma-Aldrich); trypsin (1.33 mg/mL, Sigma-Aldrich); hyaluronidase (0.67 mg/mL, Sigma-Aldrich); kynurenic acid (0.1 mg/mL, Sigma-Aldrich); and penicillin/streptomycin (50 U/mL, Thermo Fisher Scientific, Waltham, MA, USA). Tissues were incubated at 37 °C for 30 min under shaking motion. They were collected and dissociated mechanically into media hormone mixture (MHM) composed as follows: Dulbecco's modified Eagle's medium/nutrient mixture F12 (1:1, Thermo Fisher Scientific); L-glutamine (2 mM, Thermo Fisher Scientific); 0.6% D-glucose, HEPES buffer (5 mM, Sigma-Aldrich); insulin (25 μg/mL, Sigma-Aldrich); apo-transferrin (100 μg/mL, Sigma-Aldrich); progesterone (20 nM, Sigma-Aldrich); putrescine (60 μM, Sigma-Aldrich); and selenite (30 nM, Sigma-Aldrich). Additional human fibroblast growth factor-basic (bFGF, 10 ng/mL, Peprotech, Offenbach, Germany); human epidermal growth factor (EGF, 20 ng/mL, Peprotech); and heparin (2 μg/mL, Sigma-Aldrich) were added to MHM resulting in MHM-containing growth factors ($MHM_{w/GF}$). Dissociated cells were filtrated with 40-μm nylon mesh (BD Falcon, Corning, Tewksbury, MA, USA). Filtrated cells were seeded in uncoated petri dishes filled with $MHM_{w/GF}$ (2.6×10^3 cells/cm^2) and

maintained in a humidified incubator (Heraeus, Hanau, Germany, and Thermo Fisher Scientific) at 37 °C with 95% atmospheric air and 5% CO_2.

Cells proliferated for 7 days in vitro (DIV) and formed neurospheres (clusters of NSPCs). The neurospheres were collected and dissociated with a mixture of 0.25% trypsin-EDTA/HBSS (1:1, Thermo Fisher Scientific) containing recombinant DNase 1 (4.17 U/mL, Sigma-Aldrich and Takara, Kusatsu, Japan) activated by two successive incubations at 37 °C and mechanical dissociation. Dissociated NSPCs were plated in monolayers onto poly-L-ornithine-coated (Sigma-Aldrich) and laminin-coated (Thermo Fisher Scientific) wells at density 3.5×10^4 cells/cm^2 with $MHM_{w/GF}$ and incubated in a humidified incubator at 37 °C with 95% atmospheric air and 5% CO_2 for further proliferation or differentiation assays.

Proliferation Assays

DHA (Cayman Chemicals, Ann Arbor, MI, USA), AceDoPC® (CarMeN Laboratory, Villeurbanne, France) or PDX (Cayman Chemicals) was dissolved in a vehicle containing fatty acid-free BSA (1 g/L, Sigma-Aldrich), 0.1% ethanol, and α-tocopherol (70 μM, Sigma-Aldrich). DHA, AceDoPC®, or PDX solutions were incubated at 37 °C for 30 min. Two hours after monolayer plating, DHA, AceDoPC®, or PDX was added to the culture medium. The cells proliferated for 1 DIV after plating before fixing. 4 h before fixing the cells, bromodeoxyuridine (BrdU, Sigma-Aldrich and Roche, Bale, Switzerland) was added to the medium (10 μM).

Differentiation Assays

DHA, AceDoPC®, or PDX solutions were prepared as described in the proliferation assays. After monolayer plating, the cells were cultured for 1 DIV. After 1 DIV, the culture medium was changed to MHM to induce cell differentiation. DHA, AceDoPC®, or PDX was added during medium change. The cells differentiated for 4 DIV after medium change before fixing.

In Vitro Hypoxia/Ischemia

To mimic pathological conditions of stroke in vitro, we chose to apply OGD to induce hypoxia/ischemia to the cells. Upon addition of DHA, AceDoPC®, or PDX in either the proliferation or differentiation assays, the medium was changed to phosphate buffer saline solution (PBS) with growth factor (bFGF, EGF, and heparin, similarly to $MHM_{w/GF}$ for proliferation assays) or without (differentiation assays). The monolayer plated cells in PBS were put in a hypoxic incubator (Astec, Fukuoka, Japan, and Thermo Fisher Scientific) with gas composition of 1% O_2, 5% CO_2, and 94% N_2 for 30 min. After inducing OGD, the cell medium was changed back to $MHM_{w/GF}$ (proliferation assays) or MHM (differentiation assays). The OGD assays then followed the previous protocols before fixing.

Immunocytochemistry

Fixing and following immunocytochemistry was performed as described previously [45]. All steps were performed at room temperature unless stated otherwise. After either proliferation or differentiation assays, cells were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10 or 30 min, respectively. Fixed cells were rinsed with PBS. Proliferation assays required additional steps of incubation with HCl (2 N in pure water, Sigma-Aldrich) for 5 min and PBS wash. Fixed cells were permeabilized with 0.3% TritonX-100 (Sigma-Aldrich)/PBS for 5 min. After rinsing with PBS, cells were incubated for 1 h with a solution of 3% BSA/0.05% TritonX-100/PBS (named blocking buffer). Primary antibodies were added to the blocking buffer and incubated with the cells overnight at 4 °C: mouse anti-BrdU IgG (1:50, BD Biosciences, Franklin Lakes, NJ, USA) for evaluation of proliferation rates, or mouse anti-ßIII tubulin (Tuj1) IgG (1:1000, Covance, Princeton, NJ, USA) and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) IgG (1:1000, Sigma-Aldrich and Dako, Glostrup, Denmark) for evaluation of neurogenesis and astrogenesis rates, or rabbit polyclonal anti-nuclear factor erythroid 2-related factor (Nrf2) antibody (1:1000, Enzo Life Science, Farmingdale, NY, USA) for evaluation of anti-oxidative effects.

After PBS wash, secondary antibodies and DAPI (1:1000, Sigma-Aldrich) were incubated with the cells in blocking buffer for 1 h: Alexa 488-conjugated goat anti-mouse (1:400, Thermo Fisher Scientific) for proliferation assay, or Alexa 488-conjugated goat anti-mouse IgG (1:400) and Cy3-conjugated donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch, West Grove, PA, USA) for differentiation assay and study of anti-oxidative effects.

Cells were rinsed with PBS and micro-cover glasses were mounted with fluoromount (Diagnostic Biosystems, Pleasanton, CA, USA). Cells were observed with Axioplan2 (ZEISS, Oberkochen, Germany) fluorescence microscope and field pictures for quantification were taken using AxioVision software (five fields taken at random on each sample). Detection conditions and exposure times for field pictures were adapted to each filter used and samples from the same experiment were photographed at same exposure times.

Lipid Analysis

Culture media and cells from NSPC culture were separately collected. Culture media were first retrieved and cells were detached from the culture wells by treatment with 0.25%

trypsin-EDTA and 5-min incubation at 37 °C. They were stored at − 20 °C for future analysis. Lipid extraction was performed using liquid/liquid phase separation. To 500 μL of cell medium or lysed cell containing solution, 1.5 mL of MeOH/H_2O (20/80) was added. pH was adjusted to 3 using 80 μL of acetic acid. The solution was then deposited on a C18 solid-phase cartridge previously activated with MeOH and washed with H_2O and hexane. Lipids were retrieved with methyl formate and dried under N_2. Lipids were analyzed by LC/ESI/MS/MS in the negative ion mode using a Waters 2.1 × 150 mm C18 column and an acetonitrile/water gradient containing 0.1% formic acid on AB SCIEX QTRAP® 4500 system. Lipids were identified and quantified using multiple reaction monitoring. The auxiliary gas pressure was 20 psi, and the sheath gas pressure was 30 psi. The capillary voltage was set to − 4500 V. The capillary temperature was 350 °C. Lipid quantity was expressed as picomoles per microgram of proteins. Proteins were quantified using Bradford assay. Briefly, 10 μL of sample were mixed with 200 μL Bradford reagent (Sigma-Aldrich) and 790 μL distilled water. The mixture was homogenized and left at ambient temperature for 10 min. Optical density was determined on a spectrometer (Shimadzu, Kyoto, Japan) set at $\lambda = 595$ nm.

Statistics

Statistical analysis was performed using Dunnett analysis if applicable or steel analysis otherwise for multiple comparisons between groups (equivalent to unpaired Student's t test or unpaired Wilcoxon's test, respectively). Data are presented as mean ± standard error of the mean (SEM). Significance threshold was defined as $p \leq 0.05$. All statistical analyses were carried out using R software.

Results

Effects of DHA on Adult NSPCs Cultured under Physiological or OGD Conditions

High DHA Concentration Hindered Proliferation of Adult NSPCs under Physiological Conditions Only

Addition of BrdU in the proliferation assays and labeling BrdU positive cells with immunocytochemistry allowed for the evaluation of DHA effects on proliferation rates of adult NSPCs. Sample field pictures used for proliferation rates estimation are provided in Fig. 1a.

Proliferation rates were expressed as the ratio of BrdU-positive cell number over the total cell number, which was computed as the number of DAPI-positive cells. All results are expressed as percentages compared to the control group, which corresponds to the addition of vehicle only and was fixed at 100%. Proliferation rates of adult NSPCs were examined after addition of 10^{-9} M to 10^{-5} M of DHA. Addition of DHA from 10^{-9} to 10^{-6} M did not affect proliferation (data not shown) However, the highest concentration of DHA (10^{-5} M) significantly decreased the proliferation rate to 78% of control under physiological conditions (Fig. 1b).

To study DHA effects on adult NSPCs cultured under pathological conditions, OGD was induced to cells and the same evaluation of proliferation and cell count was done as previously under physiological conditions. Induction of OGD drastically changed the reaction of cells to DHA addition (Fig. 1b). Indeed, 10^{-5} M DHA concentration did not impact proliferation negatively as observed under physiological conditions.

DHA Induced Neurogenesis under OGD Conditions Only

In differentiation assays, differentiation was induced by culture media change. Specific markers Tuj1 and GFAP and counterstaining with DAPI allowed us to estimate neurogenesis rates, astrogenesis rates, and the total cell numbers, respectively. Sample field pictures used for differentiation rates estimation are provided in Fig. 2a.

Neurogenesis rates were computed as the ratio of Tuj1-positive cell number over the total cell number. Similarly, astrogenesis rates were computed with the numbers of GFAP-positive and -negative cells. All results are expressed as percentages compared to the control group, which corresponds to the addition of vehicle only and was fixed at 100%. Figure 2b, c shows the above-mentioned rates in our differentiation assays with addition of DHA, from the same concentration range as the proliferation assays (only relevant concentrations of 10^{-9} M and 10^{-8} M are shown thereafter since concentrations from 10^{-7} to 10^{-5} M did not affect differentiation). Addition of DHA did not significantly affect differentiation rates under physiological conditions. Whereas not significant, DHA tended to slightly increase neurogenesis to 115% of control at 10^{-9} M (Fig. 2b). Conversely, astrogenesis tended to be decreased at this concentration (rate lower than 80% of control group) (Fig. 2c).

Similarly to proliferation rates, differentiation rates were greatly impacted by OGD induction. DHA significantly increased neurogenesis at concentration 10^{-8} M (167% of control) (Fig. 2b). Astrogenesis was not influenced by DHA addition (Fig. 2c). Total cell number tended to increase (see supplementary Fig. S1 not significant).

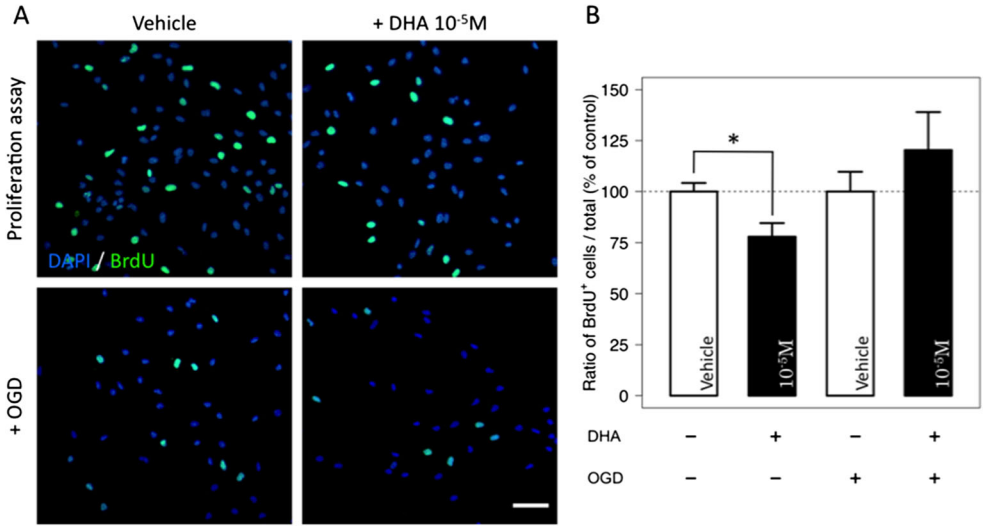

Fig. 1 **a** Immunocytochemistry and **b** proliferation rates of proliferation assays with DHA under physiological or OGD conditions. "Vehicle" refers to control condition with no DHA added to the cells. Marker used was BrdU. Cell nuclei were counterstained with DAPI. Scale bar corresponds to 50 μm. The total cell number is calculated as the number of DAPI-positive cell counted. Proliferation rates are evaluated as the ratio of BrdU positive cell number over the total cell number (**b**). 10^{-5} M DHA addition induced a decrease in proliferation under physiological conditions only. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means ± SEM. $n \geq 4$ for each group. *$p \leq 0.05$

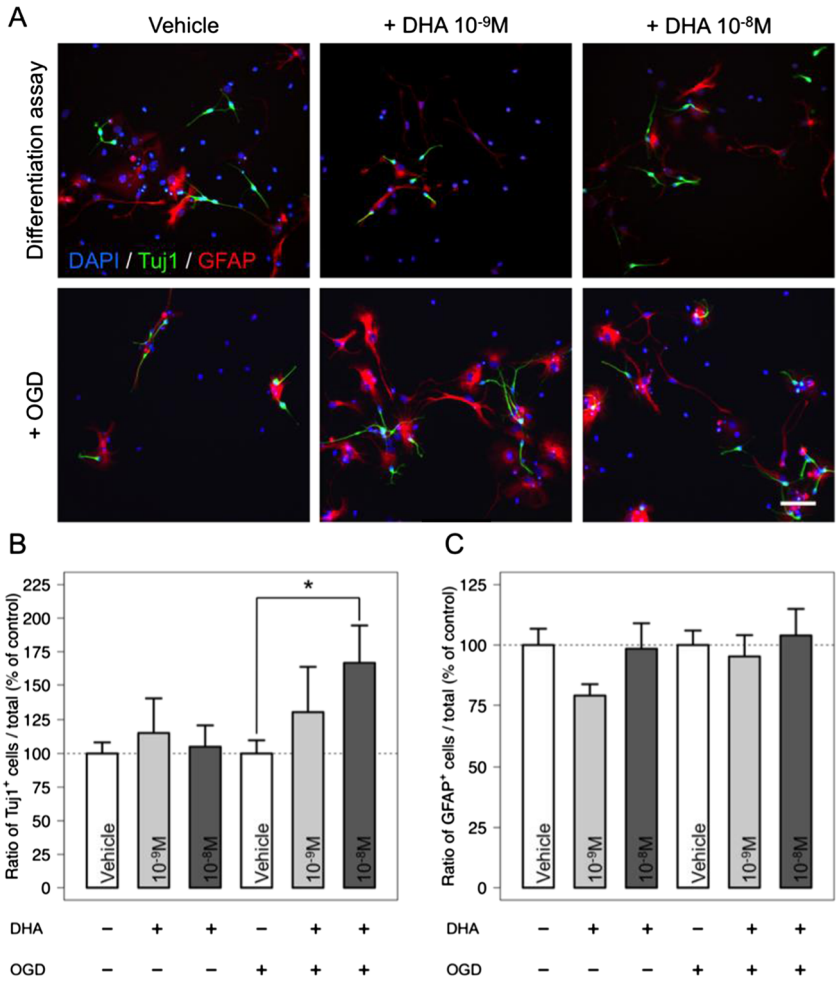

Fig. 2 **a** Immunocytochemistry, **b** neurogenesis rates, and **c** astrogenesis rates in differentiation assays with DHA under physiological or OGD conditions. "Vehicle" refers to control condition with no DHA added to the cells. Markers used were Tuj1 and GFAP. Cell nuclei were counterstained with DAPI. Scale bar corresponds to 50 μm. The total cell number is calculated as the number of DAPI-positive cell counted. Neurogenesis rates are evaluated as the ratio of Tuj1 positive cell number over the total cell number (**b**). Astrogenesis rates are evaluated as the ratio of the number of GFAP positive and negative cell number over the total cell number (**c**). No significant effect of DHA on adult NSPCs differentiation was observed under physiological conditions. Under OGD conditions, 10^{-8} M DHA addition increased neurogenesis rates significantly. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means ± SEM. $n \geq 5$ for each group

Effects of AceDoPC® on Adult NSPCs Cultured under Physiological or OGD Conditions

High Concentration of AceDoPC® Promoted Adult NSPC Proliferation under Physiological Conditions

Similarly to proliferation assays and differentiation assays with DHA, AceDoPC® was added to the cultures to evaluate the effects of DHA esterification within this acetylated structured phospholipid. We observed that high concentration of AceDoPC® (10^{-5} M) increased proliferation rates significantly, to a level of 116% of control (Fig. 3b). Equivalent high concentration of DHA decreased proliferation (Fig. 1b).

OGD induction also changed the effects of AceDoPC® on adult NSPC culture compared to physiological conditions. Little effect was observed on proliferation and 10^{-5} M AceDoPC® addition did not increase proliferation rate significantly (Fig. 3b).

Enhanced Neurogenesis with AceDoPC® Addition under Physiological Conditions and Higher Effect after OGD Induction

Results of differentiation assays with AceDoPC® addition (10^{-9} and 10^{-8} M) are shown in Fig. 4. Under physiological conditions, AceDoPC® addition increased neurogenesis to at least 110% compared to control group and the effect was significant at concentration 10^{-8} M (due to high homogeneity in this group, SEM = 1.77) (Fig. 4b). Astrogenesis rates decreased, albeit not significantly, with AceDoPC® addition (Fig. 4c).

Similarly to DHA addition under pathological conditions of cell culture, AceDoPC® showed enhanced effects on differentiation compared to the results observed under physiological condition. 10^{-9} M AceDoPC® concentration greatly and significantly increased neurogenesis up to 245% of control (Fig. 4b). Astrogenesis tended also to increase at 10^{-8} M AceDoPC® concentration albeit not significantly (Fig. 4c). However, the same concentration increased cell number significantly (see supplementary Fig. S2). Since unmarked cell ratio was not changed at this concentration (data not shown), both neurogenesis and astrogenesis might be increased at 10^{-8} M.

Effects of PDX on Adult NSPCs Cultured under Physiological or OGD Conditions

High Concentration of PDX Hindered Proliferation under Physiological and OGD Conditions

Similarly to the previous assays, we decided to evaluate the effects of DHA oxygenated derivative PDX, a molecule with anti-inflammatory, anti-aggregatory, and neuroprotective effects. Concentrations of half the previous ones were added (i.e., 0.5×10^{-5} M instead of 10^{-5} M). In Fig. 5, results for proliferation assays with PDX addition are presented. Similarly to DHA addition at 10^{-5} M, the addition of 0.5×10^{-5} M PDX significantly decreased proliferation rates (81% of control) under physiological conditions (Fig. 5b).

The same OGD induction was applied to adult NSPCs cultured with PDX. PDX effects on proliferation rates observed previously under physiological conditions were

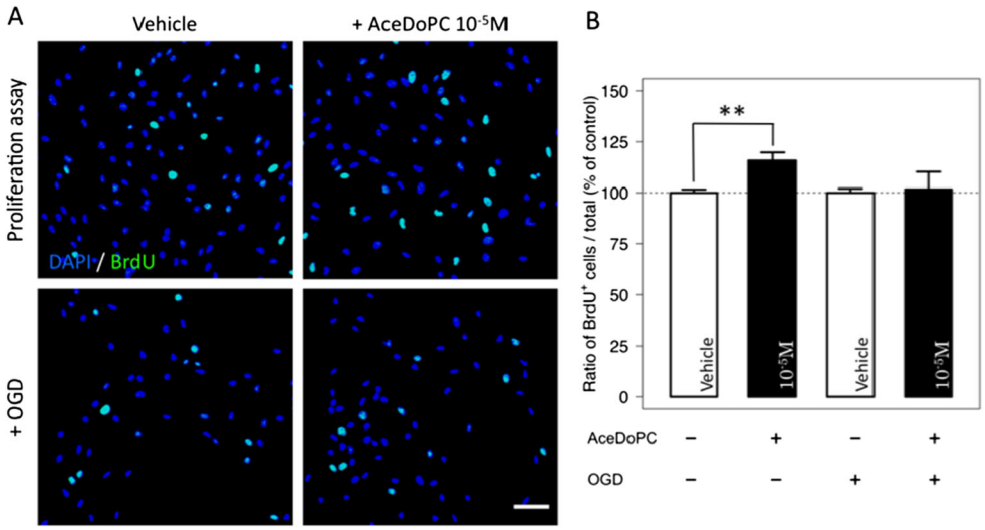

Fig. 3 **a** Immunocytochemistry and **b** proliferation rates of proliferation assays with AceDoPC® under physiological or OGD conditions. "Vehicle" refers to control condition with no AceDoPC® added to the cells. Marker used was BrdU. Cell nuclei were counterstained with DAPI. Scale bar corresponds to 50 μm. The total cell number is calculated as the number of DAPI-positive cell counted. Proliferation rates are evaluated as the ratio of BrdU-positive cell number over the total cell number (**b**). AceDoPC® increased cell proliferation at 10^{-5} M under physiological conditions. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means ± SEM. $n = 6$ for each group. $^{**}p \leq 0.01$

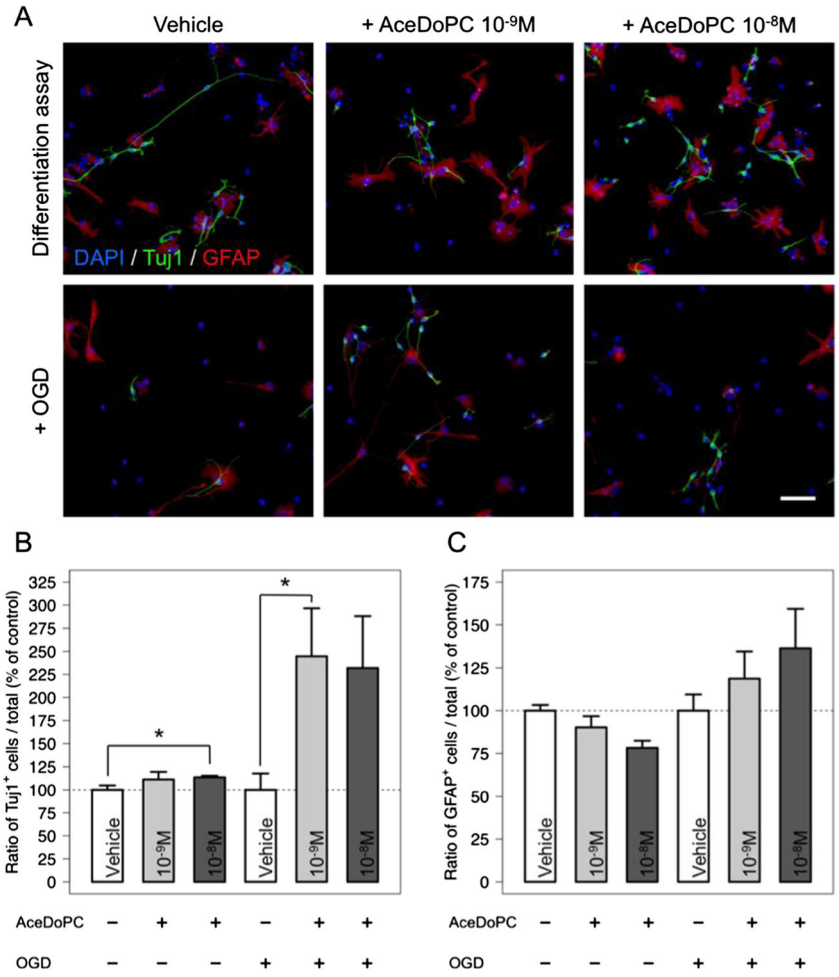

Fig. 4 **a** Immunocytochemistry, **b** neurogenesis rates, and **c** astrogenesis rates in differentiation assays with AceDoPC® under physiological or OGD conditions. "Vehicle" refers to control condition with no AceDoPC® added to the cells. Markers used were Tuj1 and GFAP. Cell nuclei were counterstained with DAPI. Scale bar corresponds to 50 μm. The total cell number is calculated as the number of DAPI-positive cell counted. Neurogenesis rates are evaluated as the ratio of Tuj1-positive cell number over the total cell number (**b**). Astrogenesis rates are evaluated as the ratio of the number of GFAP positive and negative cell number over the total cell number (**c**). A significant effect of AceDoPC on adult NSPCs neurogenesis was observed at 10^{-8} M under physiological conditions. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means ± SEM. $n \geq 6$ for each group. $^{*}p \leq 0.05$

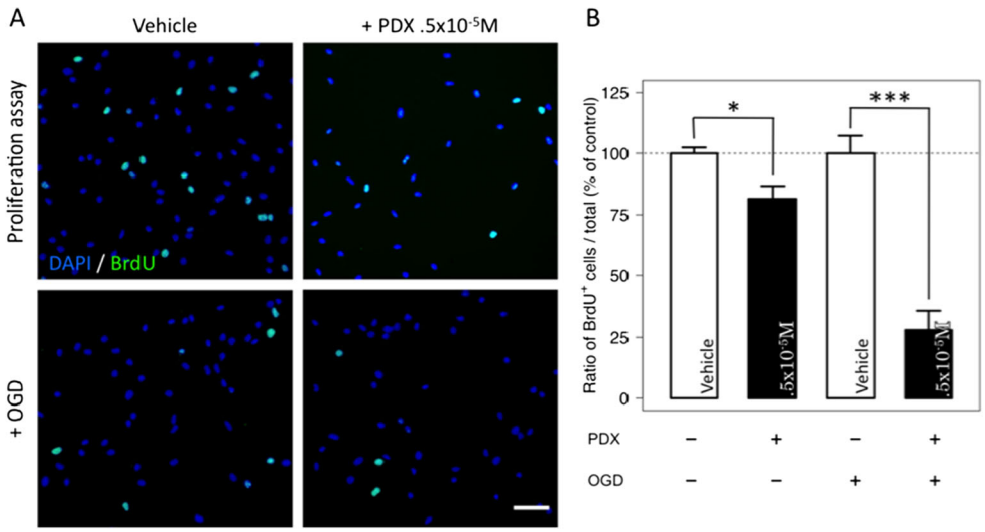

Fig. 5 **a** Immunocytochemistry and **b** proliferation rates of proliferation assays with PDX under physiological or OGD conditions. "Vehicle" refers to control condition with no PDX added to the cells. Marker used was BrdU. Cell nuclei were counterstained with DAPI. Scale bar corresponds to 50 μm. The total cell number is calculated as the number of DAPI-positive cell counted. Proliferation rates are evaluated as the ratio of BrdU-positive cell number over the total cell number (**b**). PDX decreased cell proliferation at 0.5×10^{-5} M under physiological conditions and this effect is stronger under OGD conditions. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means ± SEM. $n \geq 5$ for each group. $^{*}p \leq 0.05$; $^{***}p \leq 0.001$

increased under pathological conditions on cell cultures. Indeed, all concentrations of PDX addition tended to increase proliferation rate (0.5×10^{-9} to 0.5×10^{-6} M; data not shown) except for the highest concentration of PDX (0.5×10^{-5} M). This concentration decreased proliferation rate down to 28% of control (Fig. 5b).

PDX Effects on Differentiation of Adult NSPCs

Results of differentiation assays under physiological and OGD conditions with PDX addition are grouped in Fig. 6. No significant effect was observed on differentiation rates under physiological conditions. However, 0.5×10^{-9} M PDX addition increased the total cell number significantly (see supplementary Fig. S3). Observation of neurogenesis (Fig. 6b), astrogenesis (Fig. 6c) and unmarked cell ratio (data not shown) of this group may indicate that neurogenesis might be increased albeit not significantly. PDX tended to decrease astrogenesis rate dose dependently (Fig. 6b), which led to an increase of unmarked cell ratio rate with higher concentration of PDX (data not shown).

After OGD induction, PDX tended to enhance neurogenesis (Fig. 6b) and astrogenesis (Fig. 6c) rates, notably at concentration 0.5×10^{-8} M (166% for neurogenesis rates) while undifferentiation rate tended to decrease at this concentration (data not shown). We observed that PDX at 0.5×10^{-8} M significantly increased total cell count (see supplementary Fig. S3).

Study of the Potential Mechanisms Involved in the Effects of AceDoPC® on NSPCs

Evidence of Decrease of Oxidation and Inflammation with AceDoPC® Addition after OGD Induction

Since OGD mimics ischemic conditions in vitro, inducing OGD to our cell cultures should increase oxidative stress and activate pro-inflammatory and/or pro-apoptotic responses from the cells. To evaluate whether DHA and AceDoPC® could affect these pathways, we investigated markers of oxidation and inflammation. To this intent, differentiation assays under physiological or pathological conditions were performed. Under physiological conditions, only vehicle was

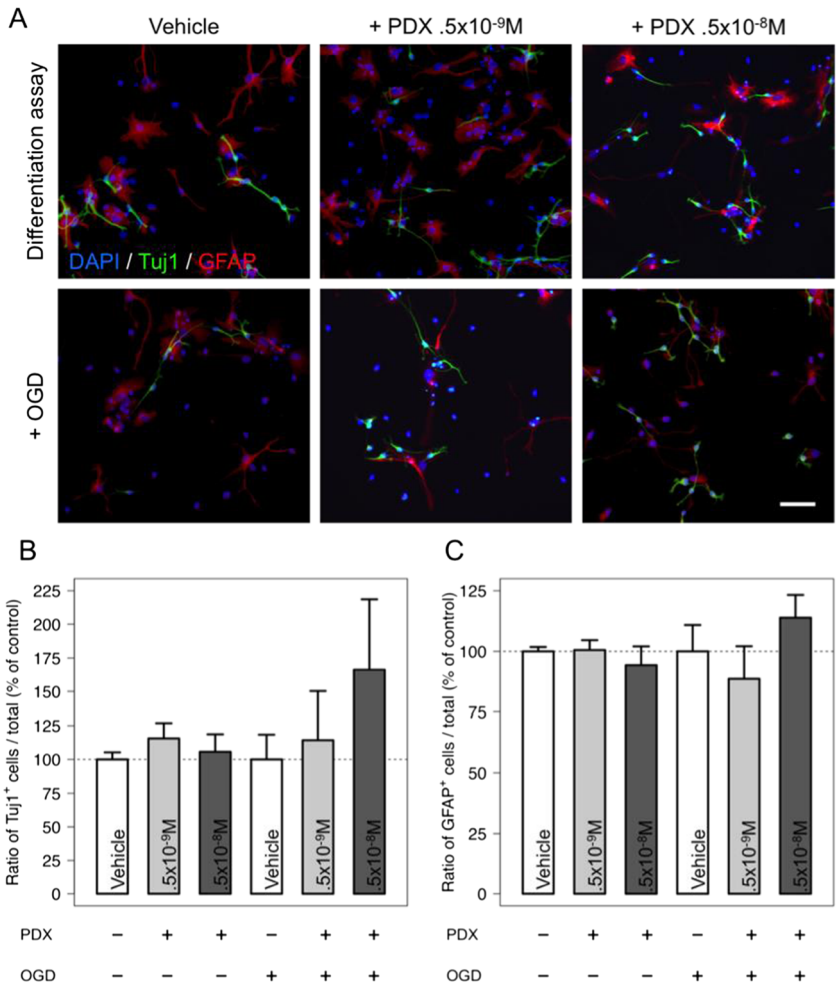

Fig. 6 **a** Immunocytochemistry, **b** neurogenesis rates, and **c** astrogenesis rates in differentiation assays with PDX under physiological or OGD conditions. "Vehicle" refers to control condition with no PDX added to the cells. Markers used were Tuj1 and GFAP. Cell nuclei were counterstained with DAPI. Scale bar corresponds to 50 μm. The total cell number is calculated as the number of DAPI positive cell counted. Neurogenesis rates are evaluated as the ratio of Tuj1-positive cell number over the total cell number (**b**). Astrogenesis rates are evaluated as the ratio of the number of GFAP-positive and -negative cell number over the total cell number (**c**). No significant effect was observed. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means ± SEM. $n \geq 5$ for each group. $^{*}p \leq 0.05$

Table 1 Identification and quantification of oxygenated lipids extracted from adult NSPC culture under different conditions

Quantification (pmoles/μg proteins)	NO OGD + vehicle	OGD + vehicle	OGD + DHA 10^{-9} M	OGD + AceDoPC 10^{-9} M
PGD_2	40.19	85.86	91.13	11.71
$PGF_{2\alpha}$	ND	467.28	507.64	82.37
8-epi-$PGF_{2\alpha}$	203.14	334.85	362.38	55.19
LTB_4	1.42	5.01	8.17	0.37
15-HETE	48.89	171.30	42.72	6.59

Lipid extraction and quantification under physiological conditions with vehicle addition (no OGD) or pathological conditions (OGD) with vehicle, 10^{-9} M DHA, or 10^{-9} M AceDoPC® addition. "Vehicle" refers to control condition with vehicle only added to the cells. Quantification was computed with internal standards and expressed as picomoles per microgram of proteins. Pool of $n = 6$ for each group

added to the cells. When cells were subjected to OGD, 10^{-9} M DHA or 10^{-9} M AceDoPC® were compared to vehicle. Culture media and cells lipids were extracted, identified, and quantified on LC/ESI/MS. The main retrieved lipids are listed in Table 1. Compared to the group cultured under physiological conditions, OGD condition induced the increase of arachidonic acid (AA, 20:4n-6)-derived mediators: prostaglandins (PG) D_2, $PGF_{2\alpha}$, leukotriene (LT) B_4, and the hydroxylated metabolite 15-HETE, and 8-epi-$PGF_{2\alpha}$. AceDoPC® addition at 10^{-9} M reduced all these lipids' concentrations. 10^{-9} M DHA did not show the same decreasing effect except for 15-HETE. Oxygenated metabolites derived from DHA were also identified: 17-hydroxy-docosahexaenoic acid (HDoHE), 14-HDoHE, 11-HDoHE, 7-HDoHE, and 4-HDoHE (not shown).

Evidence of Anti-oxidative Signaling Pathway Activation in Response to AceDoPC®

In the AceDoPC® neuroprotective properties, we investigated by immunocytochemistry the Nrf2 pathway for two groups of cells cultured under OGD and with or without 10^{-9} M AceDoPC®. Using Nrf2-antibody, we could observe for each group that AceDoPC® activated Nrf2 production in adult NSPCs compared to the cells subjected to OGD and added with vehicle only (Fig. 7).

Whereas these results are preliminary, we believe that this tendency could imply the potential mechanisms underlying neuroprotective effects of AceDoPC®.

Discussion

In this work, we show the potential of AceDoPC®, a structured phospholipid that specifically targets the brain with DHA as a neuroprotective agent and neurogenesis inducer on adult NSPCs. Our motivation for exploring the effects of brain targeting with AceDoPC® on a model of neurogenesis and ischemia in vitro stems from the fact that deficiency of DHA is associated with neurodegenerative diseases and an exogenous supply of DHA is necessary and recommended to ensure brain functions. Due to this requirement of DHA, new therapeutic strategies to bring it to the central nervous system are crucial. DHA has been previously shown to increase the number of neurons derived from embryonic NSPCs in non-pathological conditions which is of particular importance since one therapeutic approach to neurodegenerative diseases is to promote neurogenesis.

Our study shows that esterification of DHA within AceDoPC® prevents decreased proliferation of adult NSPCs that can be observed after addition of the highest concentration of DHA (10^{-5} M). Sakayori et al. [39] previously showed that DHA addition at such concentration range to embryonic NSPCs

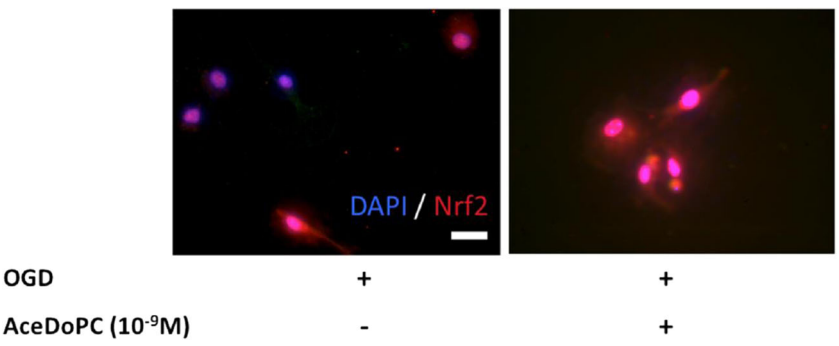

Fig. 7 Immunocytochemistry with Nrf2 following differentiation assays under OGD or OGD + AceDoPC conditions. "Vehicle" refers to control condition with vehicle only added to the cells. Marker used was Nrf2-antibody and cells were counterstained with DAPI. Scale bar corresponds to 50 μm

decreased proliferation rates and also increased proportion of active caspase 3-positive cells, caspase 3 being a marker of cell apoptosis. The authors concluded that high concentrations of DHA was toxic for NSPCs due to lipid peroxidation converting DHA to oxidized mediators, thus inducing oxidative stress to the cells. Such results were also observed on cell viability of embryonic NSPCs cultured with 10^{-5} M DHA [46]. One study of rats fed with diets containing high levels of DHA showed that lipid peroxidation was higher in plasma, liver, and kidney with notably decreased levels of the anti-oxidant α-tocopherol [47]. The same effect was observed in adult NSPCs cultured with PDX at 0.5×10^{-5} M. PDX being a dioxygenated derivative of DHA, high levels of PDX might also prove toxic to cell proliferation. NPD1, a PDX isomer, was proven to protect human retinal pigment epithelial cells from oxidative stress but tested concentrations were lower than 10^{-5} M [48]. Conversely, no such effect was observed with addition of 10^{-5} M AceDoPC®. This might indicate that esterified DHA would be less peroxidizable in response to oxidative stress, allowing AceDoPC® to exert a neuroprotective property. Protection against lipid peroxidation of DHA esterified within phospholipids was observed in mice fed with DHA-containing diets under different forms [49], and the same results were obtained in rats [50]. High concentration of AceDoPC® also showed an increase of proliferation instead. Adding to its putative anti-oxidative property, AceDoPC® might also be an inducer of proliferation.

Cells subjected to OGD, an in vitro condition mimicking ischemia by reducing oxygen and glucose availability to the cells [21], showed different responses to DHA, PDX, or AceDoPC® addition. Previously described decreased proliferation due to high DHA concentration was not observed after OGD induction. Since DHA also showed anti-inflammatory and anti-oxidative properties due to its conversion into derived mediators, notably protectins [30, 31, 51–53], DHA added to culture media could have been metabolized by the cells to counteract pathological culture conditions. There was no change in the effects of high concentration of PDX (0.5×10^{-5} M) on proliferation of adult NSPCs after OGD induction. Proliferation rates were even lower than under physiological conditions. This result could suggest that PDX does involve higher oxidative stress on the cells and that PDX cannot be metabolized into other mediators with beneficial properties, contrary to DHA. However, PDX effects on lipid metabolism were not studied in this work and remain to be investigated.

We demonstrated that neurogenesis rates were greatly influenced by OGD induction, and 10^{-8} M DHA significantly increased neurogenesis of adult NSPCs. Although small increase of neurogenesis was observed under physiological conditions of culture, the effect was much higher after OGD. Astrogenesis rates were not affected by DHA. Similar results were observed on embryonic NSPCs under physiological conditions [39, 42, 54]. Adult NSPCs and embryonic NSPCs differ by the preferred fate of differentiation of the stem cell. Embryonic NSPCs of earlier stage are qualified as neurogenic because of their tendency to become neurons, which is primary for the earliest stages of brain development. Later-stage NSPCs tend to become astrocytes, shifting the NSPCs from neurogenic to gliogenic qualification, but they can also produce neurons [55, 56]. DHA might shift the tendency of differentiation into neurons instead of astrocytes. This is in accordance with the observed high levels of DHA in the earliest stages of brain development [57–59], making DHA a key factor of neurogenesis.

AceDoPC® also increased neurogenesis rates at concentrations of 10^{-9} and 10^{-8} M. Neurogenesis was increased under physiological conditions but OGD induced a greater increase in neurogenesis levels by AceDoPC®. Since neurogenesis induction happened at lower concentrations of AceDoPC® and higher levels compared to DHA, we may assume that either AceDoPC® allows DHA to be more readily available for cells or that AceDoPC® have unique properties allowing enhancement of neurogenesis. In agreement with our previous data [19], we observed that DHA, applied as AceDoPC®, incorporated into the cells (supplementary Fig. S4). AceDoPC® is a preferred carrier of DHA through the BBB compared to unesterified DHA [16, 19, 23]. This suggests that AceDoPC® crosses cell membrane more easily than unesterified DHA and this could explain the neurogenesis enhancement at lower concentration of AceDoPC®. Another suggestion might be that the acetyl group of AceDoPC® at *sn*-1 position has beneficial properties on neurogenesis.

Although 10^{-8} M AceDoPC® did not show a statistically significant effect on neurogenesis or astrogenesis, we could note that the total cell number in the differentiation assay was significantly higher at this concentration. Unmarked cell ratio was not affected by AceDoPC®, so this could suggest that increases in both neurogenesis and astrogenesis influenced the total cell number in our cultures. This is of particular interest since no effect of DHA or DHA metabolites on astrogenesis have been reported yet to our knowledge. Moreover, glial cells were shown to be inducers of neurogenesis [60, 61]. Therefore, putative astrogenesis induction by AceDoPC® might represent a beneficial effect on neurogenesis induction. Our results also showed that cell cultures under pathological conditions through OGD induction led to increased oxidative and pro-inflammatory markers. 10^{-9} M AceDoPC® decreased the level of arachidonic acid (AA) metabolites involved in oxidative stress or inflammation process (e.g., 8-epi-$PGF_2\alpha$, PGD_2, PGF_2, LTB_4, and 15-HETE). OGD is a condition mimicking ischemia in vitro, meaning induction of hypoxia and ischemia to the cell cultures. Such conditions were shown to activate the AA metabolism cascade with upregulation of cyclooxygenase-2 [62, 63]. 5-lipoxygenase expression is also increased and the concentration of leukotrienes is elevated in the brain after cerebral ischemic injury [64, 65]. 15-HETE level, synthesized by 15-

lipoxygenase, is also higher in brain of rat exposed to ischemia [66, 67]. AA metabolites are considered as the main actors of the pro-inflammatory response of cells against damage and are therefore considered as pro-inflammation markers, although recent studies highlighted their potential use as protective agents [68]. 8-epi-$PGF_{2\alpha}$, a marker of oxidative stress, is elevated in patients who experienced stroke [69] and in rats subjected to oxidative stress following traumatic brain injury [70]. Moreover, 8-epi-$PGF_{2\alpha}$ induced cell death on cultures of endothelial cells [71].

Analysis of Nrf2 expression by immunocytochemistry showed that Nrf2 pathway was better activated by AceDoPC®. Nrf2 is involved in the anti-oxidative response of cells against oxidative damage by upregulation of enzymes such as heme oxygenase 1 [22]. Addition of DHA also showed the same activation of Nrf2 (not shown) but no decrease of oxidation markers. Overall, these results suggest that AceDoPC® exhibits neuroprotective effects on cells in part by enhancing the anti-oxidative capacity of cells. AceDoPC® effects might be due to either DHA esterified at the *sn*-2 position of the phospholipid and/or the acetyl group esterified at the *sn*-1 position. This neuroprotective effect might affect cell condition and allow maintenance of newly differentiated neurons, thus enhancing neurogenesis in our experiments. Although preliminary, these results are promising and will help us identifying which pathways to investigate in future studies.

If high concentration of PDX decreases proliferation rates of NSPCs, we could observe that lower concentrations of PDX increased the total cell number in the differentiation assays under physiological and pathological conditions. Although seemingly contradictory, this effect was not observable in the proliferation assays. This could be explained by the composition of culture media in each assay type. Proliferation assay culture medium contains growth factors that maintain the cells in a proliferative state while culture medium of differentiation assay does not contain such factors. This effect is still observed in cell culture under pathological conditions but at higher concentrations of PDX. Since no significant effect was observed in differentiation rates, PDX influence on adult NSPCs remains unclear and to be investigated. Another possibility is that PDX promotes survival of newly differentiated cells. Yokose et al. previously showed that important cell death rate occurs during a certain critical period following initial cell proliferation in hippocampal slice cultures from rat brain [72]. Such results are in accordance with previous studies [73, 74]. PDX might play a role in the maintenance of newly generated neurons facing this critical period of postmitotic cell death. DHA, PDX precursor, and NPD1, PDX isomer, notably proved to be anti-apoptotic in ischemic brain tissues from rats [75] and in vitro studies of human neurons and glia cultures [42], respectively. Therefore, PDX might also have anti-apoptotic effects on adult NSPCs cultured under pathological conditions.

Conclusion

We conclude that DHA and AceDoPC® are effective neurogenesis inducers on adult NSPCs, notably when the cells are subjected to pathological conditions. Moreover, AceDoPC® effects were induced at lower concentration compared to unesterified DHA. This neurogenesis induction might be paralleled with neuroprotection due to putative anti-oxidative effects of AceDoPC®. These results lead toward a better understanding of AceDoPC® effects on the brain after its passage through the BBB and hint at the possible use of AceDoPC® and DHA as a treatment or prevention of stroke for preservation and regeneration of stressed cells.

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