

# DHA protects PC12 cells against oxidative stress and apoptotic signals through the activation of the NFE2L2/HO-1 axis

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**Abstract.** Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid, derived mainly from fish oil. It is well known that DHA is present in high concentrations in nervous tissue and plays an important role in brain development and neuroprotection. However, the molecular mechanisms underlying its role remain to be fully elucidated. In this study, to enhance our understanding of the pathophysiological role of DHA, we investigated the possible neuroprotective mechanisms of action of DHA against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced oxidative damage in a rat pheochromocytoma cell line (PC12). Specifically, we evaluated the viability, oxidation potential, and the expression and production of antioxidant/cytoprotective enzymes, and eventual apoptosis. We found that pre-treatment with DHA (24 h) protected the cells from H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. In particular, pre-treatment with DHA: i) Antagonized the consistent decrease in viability observed following exposure to H<sub>2</sub>O<sub>2</sub> for 24 h; ii) reduced the high levels of intracellular reactive oxygen species (ROS) associated with H<sub>2</sub>O<sub>2</sub>-induced

oxidative stress; iii) increased the intracellular levels of enzymatic antioxidants [superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)] both under basal conditions and following H<sub>2</sub>O<sub>2</sub> exposure; iv) augmented the intracellular levels of reduced glutathione (GSH) and ascorbic acid, while it reduced the malondialdehyde (MDA) levels under conditions of oxidative stress; v) upregulated the expression of nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) and its downstream target protein, heme-oxygenase-1 (HO-1); and vi) induced an anti-apoptotic effect by decreasing Bax and increasing Bcl2 expression. These findings provide evidence suggesting that DHA is able to prevent H<sub>2</sub>O<sub>2</sub>-induced oxidative damage to PC12 cells, which is attributed to its antioxidant and anti-apoptotic effects via the regulation NFE2L2/HO-1 signaling. Therefore, DHA may play protective role in neurodegenerative diseases associated with oxidative stress.

## Introduction

A number of studies have indicated that the brains of patients with neurodegenerative disease (such as brain stroke, Parkinson's and Alzheimer's diseases) is subjected to increased oxidative stress, induced by an imbalanced redox states (1,2). The excessive generation of reactive oxygen species (ROS) and the dysfunction of the antioxidant system are widely considered to be responsible for neuronal degeneration (3). Neurodegenerative processes are mostly mediated by ROS, as well as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide anion and hydroxyl radicals, which are generated as products of normal or altered metabolic processes that utilize molecular oxygen. ROS, produced by oxidative stress-caused mitochondrial damage, can damage the proteins, nucleic acids and polyunsaturated fatty acids of cell membranes. The consequent alterations of cellular functions and membrane integrity lead to cellular apoptosis or necrosis (4).

Antioxidant therapy has been suggested for the prevention and treatment of neurodegenerative diseases; in particular, an increased dietary intake of polyunsaturated fatty acids (PUFAs) is considered an efficient strategy for the prevention of neurological disorders (5). In this regard, the use of

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**Abbreviations:** DHA, docosahexaenoic acid; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme-oxygenase-1; ARE, antioxidant response element; GSH, reduced glutathione; Keap1, Kelch-like ECH-associated protein 1; NQO1, NAD(P)H:quinone oxidoreductase 1; CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase

**Key words:** docosahexaenoic acid, oxidative stress, apoptosis, nuclear factor (erythroid-derived 2)-like 2, heme oxygenase-1

docosahexaenoic acid (DHA), the main  $\omega$ -3-PUFA in the mammalian brain, plays a crucial role in the development, function and protection of brain neurons (6). In fact, several studies have highlighted that the consumption of DHA, but not that of other n-3 fatty acids, is associated with a reduced risk of stroke and cognitive decline or dementia (7,8). Although the molecular mechanisms regarding its efficacy in the prevention of neurodegenerative disease are not yet entirely clear, it is considered that DHA protects the cells from oxidative stress by the synthesis of factors responsible for cellular defense mechanisms (9). Among these elements, nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) represents one of the most important protective agents against oxidative stress (10-12). The activation of NFE2L2 induces the transcriptional regulation of various antioxidant enzymes, such as heme-oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1), as well as the modulation of apoptosis-related genes (13).

Within this framework, the present study was designed with an aim to obtain a better understanding of the mechanisms underlying the cellular protective effects of DHA. Therefore, using an *in vitro* H<sub>2</sub>O<sub>2</sub>-induced PC12 cell oxidative damage model, we examined the associations among the antioxidant effects of DHA pre-treatment and ROS production, mitochondrial membrane potential ( $\Delta\Psi$ m), apoptosis and the activation of the NFE2L2/antioxidant response element (ARE) signaling pathway, in order to provide a possible explanation for the protective effects of DHA.

## Materials and methods

**Cell line, culture and treatments.** PC12 cells (a cellular line of rat pheochromocytoma from Sigma-Aldrich Co., St. Louis, MO, USA. Cat. no. 88022401) were cultured in a 5% CO<sub>2</sub> atmosphere at 37°C in RPMI with 10 mM HEPES, 1.0 g/l glucose, 3.7 g/l NaHCO<sub>3</sub>, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal calf serum and 15% horse serum. Once grown to 85% confluence, the cells were subcultured at an appropriate density according to each experimental procedure.

The cells were treated with a physiological concentration (60 µM) of DHA (purchased from Sigma-Aldrich Co.) 24 h prior to and during H<sub>2</sub>O<sub>2</sub> exposure (for a further 24 h). For the control groups, medium or H<sub>2</sub>O<sub>2</sub> alone were used where appropriate. DHA was dissolved in 99.5% ethanol to prepare a 500 mM stock solution; prior to use, DHA was complexed (1:2) to bovine serum albumin (BSA) and diluted in RPMI to the required concentration. The concentration of H<sub>2</sub>O<sub>2</sub> used was 300 µM; since, in a previous series of dose-response experiments, described in detail in (14), we demonstrated that after 24 h, H<sub>2</sub>O<sub>2</sub> resulted in a reduction of the viability of PC12 cells of 50% at this concentration. At the end of all the experiments, the cells were collected and stored at 37°C (unless otherwise stated) for the analytical experiments.

**Measurement of cell viability and intracellular ROS production.** For the determination of cell viability and ROS production, the PC12 cells were plated in 96-well plates at a density of 10,000 cells/well and incubated with DHA 24 h prior to exposure to H<sub>2</sub>O<sub>2</sub>. Cell survival was evaluated by the 3-[(4,5-dimethylthiazol-2-yl)-5,3-carboxymethoxyphenyl]-2-(4-sulfophenyl)-2H tetrazolium,

inner salt reduction assay (MTS). The MTS assay (Promega srl, Padova, Italy) is a sensitive measurement of the normal metabolic status of cells, which reflects early cellular redox changes. The intracellular soluble formazan produced by the cellular reduction of the MTS was determined by recording the absorbance of each 96-well plate using the automatic microplate photometer (BioTek™ Elx800 -Box 998; BioTek Instruments, Winooski, VT, USA) at a wavelength of 490 nm. The morphological features of the PC12 cells subjected to the different treatments were analyzed and photographed by phase-contrast microscopy (Nikon eclipse ts100 microscope; Nikon, Tokyo, Japan), at a magnification of x20.

The detection of ROS production was performed using the 2',7'-dichlorofluorescein diacetate (DCFDA)-Cellular ROS Detection Assay kit (Abcam, Cambridge, UK). DCFDA is initially non-fluorescent and is converted by oxidation to DCF, a highly fluorescent compound. DCF was quantified using a CytoFluor Multiwell Plate Reader (Victor 3 Wallac 1420; Perkin Elmer Waltham, MA, USA), with 485 nm excitation and 538 nm emission filters. ROS production was expressed as the fluorescence intensity and presented as a percentage relative to the control.

**Measurement of enzymatic and non-enzymatic antioxidant levels.** The cell cultures (1x10<sup>6</sup>) were washed twice in phosphate buffered saline (PBS, pH 7.4) and subsequently centrifuged at 1,890 x g for 10 min at 4°C, producing a cell pellet and the supernatant. Cell pellets were processed for the determination of the enzymatic activity of total glutathione peroxidase (GSH-Px), catalase and total superoxide dismutase (SOD), by adding 500 µl/10<sup>6</sup> cells of a hypotonic buffer containing 15 mM KCl + 1 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. After vigorous vortexing for 60 sec, the cell suspensions were sonicated at 4°C for 3 cycles of 20 sec each with 20 sec intervals between each pulse of sonication. Subsequently, the samples were centrifuged at 20,690 x g for 15 min at 4°C to remove any cell debris. Enzymatic activities were performed spectrophotometrically (using an Agilent 89090A spectrophotometer; Agilent Technologies, Santa Clara, CA, USA) on cell lysates. Total GSH-Px activity was assayed as previously described (15). Briefly, 1 ml of reaction mixture had the following composition: 0.1 M Tris-HCl, pH 8.0, 4 mM EDTA, 2 mM reduced glutathione (GSH), 1 IU glutathione reductase, 0.3 mM NADPH and 10 µl of cell extract. Following 10 min of incubation at 37°C, the reaction began by the addition of 70 µM tert-butyl hydroperoxide (TBH) and monitored spectrophotometrically (Agilent 89090A) at 340 nm by following for 10 min NADPH oxidation; blank without TBH and additional blank without cell lysate were used. The millimolar extinction coefficient of 6.3x10<sup>-3</sup> of NADPH was used for the calculation. Catalase activity was examined as previously described (15); the blank reaction mixture (1 ml final volume) was composed by 10 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.4 and H<sub>2</sub>O<sub>2</sub> 10 mM; the reaction began by the addition of 5 µl of cell extract and monitored spectrophotometrically (Agilent 89090A) at 240 nm by following H<sub>2</sub>O<sub>2</sub> dismutation. The millimolar extinction coefficient of 3.94x10<sup>-3</sup> of H<sub>2</sub>O<sub>2</sub> was used for the calculation. Total SOD activity was analyzed as previously described (16). The reaction mixture (1 ml final volume) was formed by 50 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.8, 10 µM oxidized cytochrome c, 1.25 mM xanthine, 0.006 µM xanthine oxidase and 4 mM sodium azide (to inactivate

catalase). The reaction began by the addition of 20  $\mu$ l of cell extract and monitored spectrophotometrically (Agilent 89090A) at 550 nm following the cytochrome *c* reduction. The millimolar extinction coefficient of  $8.4 \times 10^{-3}$  of oxidized cytochrome *c* was used for the calculation. All enzymatic activities were normalized for the protein content determined by Bradford assay (17) and expressed as IU/mg of protein (1 IU=1  $\mu$ mol substrated consumed/min). In a different set of experiments, cell pellets, obtained as described above, were treated for the determination of non-enzymatic antioxidant levels with a precipitating solution composed of CH<sub>3</sub>CN 75% and KH<sub>2</sub>PO<sub>4</sub> 25% (10 mM) at pH 7.4, and then centrifuged at 20,690  $\times$  g for 15 min at 4°C. The supernatants were collected and subjected to 2 chloroform washings in order to obtain an upper aqueous phase that was directly injected onto the HPLC. Each sample was analyzed to determine the concentration of GSH, ascorbate, malondialdehyde (MDA), nitrites and nitrates (NO<sub>2</sub> and NO<sub>3</sub>) according to an ion-pairing HPLC method previously set up in our laboratories (18,19). All concentration values were normalized with respect to the protein amount and expressed as nmol/mg protein (17).

**Detection of apoptotic cells by Annexin V.** The cells were seeded at a density of  $1 \times 10^6$  cells/well on a 24-well plate and incubated with DHA 24 h prior to the exposure to H<sub>2</sub>O<sub>2</sub> (300  $\mu$ M for 24 h). The medium was then aspirated, and the cells were washed with PBS. After the cells were incubated at room temperature, for 15 min in a dark room with Annexin V-FITC (TACS Annexin V kit; Trevigen, Gaithersburg, MD, USA). The cells were then examined under a fluorescence microscope (Nikon Eclipse TE300 Inverted Fluorescence Microscope; Nikon) with a  $\times 20$  objective lens.

**Detection of  $\Delta\Psi_m$ .**  $\Delta\Psi_m$ , an early marker of the induction of apoptosis, was assessed using the Mitolight™ Apoptosis Detection kit (APT142; Chemicon International, Temecula, CA, USA). The cells ( $4 \times 10^4$  cells/well in a PLL-coated black-bottom 96-well plate) (treated as described above) were incubated with 100  $\mu$ l of prediluted Mitolight™ dye solution for 15 min (37°C, 5% CO<sub>2</sub>, 95% air). In healthy cells, the dye accumulates and aggregates in the mitochondria, giving off a bright red fluorescence ( $\lambda_{em}$ =585 nm). In apoptotic cells with an altered  $\Delta\Psi_m$ , the dye in monomeric form remains in the cytoplasm, fluorescing green ( $\lambda_{em}$ =530 nm) and thus providing a ready discrimination between apoptotic and non-apoptotic cells. The fluorescence intensities were measured with a Victor multilabel plate reader (PerkinElmer, Waltham, MA, USA) at an excitation wavelength 485 nm and an emission wavelength 530 nm to monitor apoptotic cells and 585 nm for healthy cells, respectively. The data were expressed as a fluorescence ratio (585/530) representative of the arbitrary  $\Delta\Psi_m$ . The cells were also observed using a Nikon Eclipse TE300 Inverted Fluorescence Microscope (Nikon) (data not shown).

**RNA isolation and semi-quantitative PCR.** Total RNA was isolated using the SV Total RNA Isolation System (Promega srl). The RNA concentration was evaluated by spectrophotometric reading at 280 and 260 nm. Total RNA was used for first strand cDNA synthesis with the HyperScript, First strand Synthesis

kit and Oligo-dT, as random primer (GeneAll Biotechnology Co. Ltd., Seoul, Korea). PCR was performed with about 150 ng of cDNA using PCRBI Classic Taq (PCR Biosystems Ltd., London, UK). The following primer sequences were used for amplification: Actin  $\beta$  ( $\beta$ -actin) forward, 5'-TGC TATGTTGCCCTAGACTTCG-3' and reverse, 5'-GTTGGC ATAGAGGTCTTTACGG-3' (240 bp); Bax forward, 5'-GCA GGGAGGATGGCTGGGGAG-3' and reverse, 5'-TCCAGA CAAGCAGCCGCTCACG-3' (352 bp); Bcl2 forward, 5'-CAC CCCTGGCATCTTCTCCT-3' and reverse, 5'-GTTGACGCT CCCACACACA-3' (349 bp); NFE2L2 forward, 5'-GCC AGCTGA ACTCCTTAGAC-3' and reverse, 5'-GATTCG TGCACAGCAGCA-3' (466 bp); and HO-1 forward, 5'-GAA ACAAGCAGAACCCAGTC-3' and reverse, 5'-AGAGGT CACCCAGGTAGCG-3' (225 bp).

The experimental protocols, in accordance with the conditions reported in the literature (14,20) and slightly modified, were as follows: An initial denaturation for 3 min at 95°C; amplification for 21 ( $\beta$ -actin), 20 (NFE2L2), 30 (HO-1) and 40 cycles (Bax and Bcl2) of denaturation, 15 sec, 95°C, annealing: 15 sec, at 61°C ( $\beta$ -Actin), 58°C (NFE2L2), 55°C (HO-1), 60°C (Bax), 59°C (Bcl2) and elongation at 72°C for 1 min; and a final elongation for 10 min at 72°C. The PCR products were then analyzed by 1.5% agarose gel electrophoresis in TBE1X buffer. Image acquisition and product analysis were made using Bio-Rad imaging systems with Quantity One® 1-D analysis software.

**Bax, Bcl2, NFE2L2 and HO-1 intracellular detection.** The detection of intracellular Bax, Bcl2 and NFE2L2 proteins was performed using colorimetric cell-based ELISA kits from Assay Biotechnology (Sunnyvale, CA, USA) for Bax (cat. no. CB5066) and for Bcl2 (cat. no. CB5068) and from LSBio/LifeSpan Biosciences, Inc. (Seattle, WA, USA) for NFE2L2 (cat. no. LS-F2192). In brief, the cells were seeded at  $3 \times 10^4$  cells/well in a 96-well dish and treated according to the experimental protocol; at the end of the experiment, the cells were fixed with 4% formaldehyde. Successively, quenching buffer, blocking buffer and finally primary antibodies (rabbit anti-Bax, rabbit anti-Bcl2, rabbit anti-NFE2L2 according to the experimental design, or mouse anti-GAPDH; the latter used as a positive internal control) were added followed by incubation overnight at 4°C. All primary antibodies were diluted 1:100 according to the Technical Manual (the antibodies were included with the kit). Following this overnight incubation, the secondary antibodies (included with the kit; anti-rabbit IgG for Bax, Bcl2 and NFE2L2; Anti-mouse IgG for GAPDH) conjugated to peroxidase were added and subsequently the samples were read with a microplate reader (BioTek™ Elx800 -Box 998; BioTek Instruments) [optical density (OD) at 450 nm]. The positive controls were performed in the same plates with the target experiments. All values obtained, normalized to GAPDH OD450, were expressed as a percentage relative to the control (untreated cells).

For the intracellular detection of HO-1, the PC12 cells were examined using a kit sandwich ELISA (Abcam, Cambridge, UK, cat. no. ab213968). The ELISA was performed according to the manufacturer's protocol. Briefly,  $1 \times 10^6$  cells, after treating, were lysed (using 1 ml extraction reagent, provided with the kit, supplemented with protease inhibitors) and

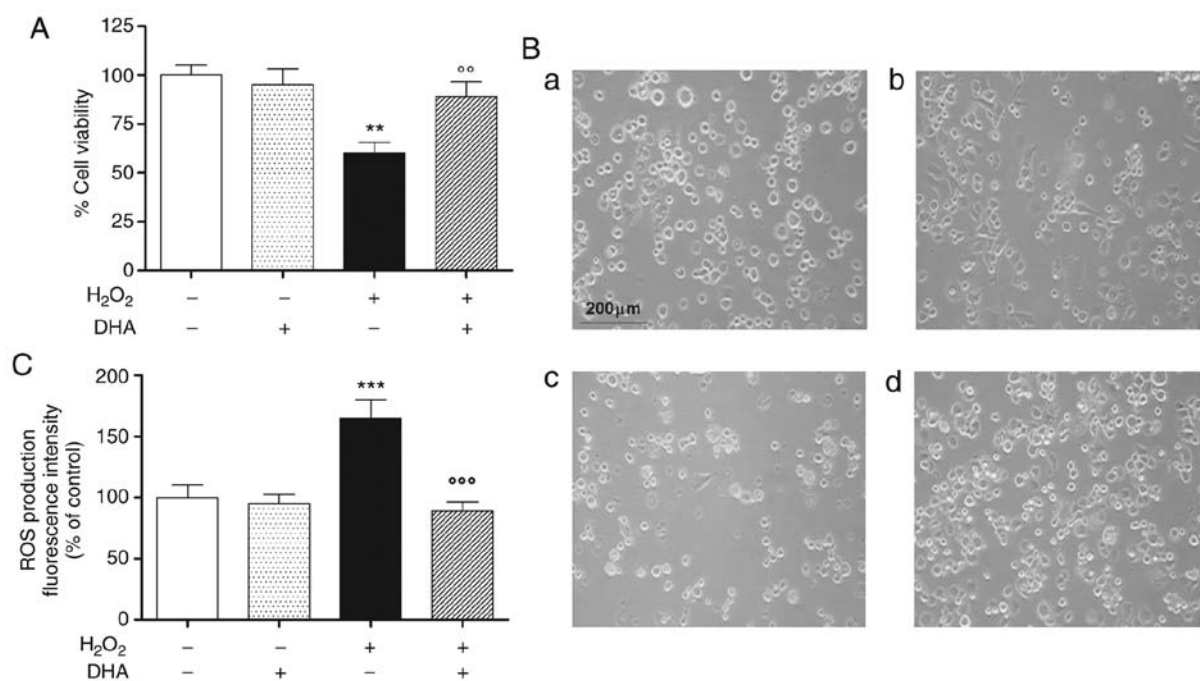


Figure 1. (A) PC12 cell viability following pre-treatment with 60  $\mu$ M DHA (for 24 h) under baseline conditions and under H<sub>2</sub>O<sub>2</sub> stimulation. (B) Morphology of PC12 cells in the same experimental paradigm observed by phase-contrast microscopy: Panel a, controls; panel b, cells treated with DHA alone; panel c, cells exposed to H<sub>2</sub>O<sub>2</sub> alone; panel d, cells pre-treated for 24 h with DHA and successively with H<sub>2</sub>O<sub>2</sub>. (C) ROS production (expressed as fluorescence intensity) in PC12 cells following pre-treatment with 60  $\mu$ M DHA (for 24 h) under basal conditions and after H<sub>2</sub>O<sub>2</sub> stimulation. For the experimental details, please see the Materials and methods section. Data are expressed as a percentage relative to the untreated cells (control=100%) and are presented as the means  $\pm$  SEM of 7 replicates per group. \*\*P<0.01 and \*\*\*P<0.001 vs. control; \*\*P<0.01 and \*\*\*P<0.001 vs. H<sub>2</sub>O<sub>2</sub> alone, respectively. DHA, docosahexaenoic acid.

centrifuged at 10,000  $\times$  g, at 4°C for 10 min. The collected supernatants, following protein measurement and normalization, were plated into plates precoated (with monoclonal anti-rat HO-1 antibody, ready to use). HO-1 immobilization was detected with a specific polyclonal antibody and successively with a secondary antibody (Rat HO-1, ready to use) conjugated to the peroxidase. The intensity of the color was measured on a microplate reader (BioTek™ Elx800 -Box 998; BioTek Instruments) at 450 nm. To generate the standard curve, provided rat HO-1 standard (5  $\mu$ g/ml) was stepwise diluted (25, 12, 5, 6, 5, 3, 13, 1, 56, 0, 78, 0, 39 and 0 ng/ml) and the readers were plotted (using a linear scale) and thus: Concentrations (ng/ml) on the x-axis and absorbance measurements on the y-axis. HO-1 concentrations from the samples were quantified by interpolating the absorbance readings from a standard curve. The data were expressed as a percentage relative to the control (untreated cells).

**Statistical analysis.** Each experiment was repeated at least 3 times, separately. All the results are presented as the means  $\pm$  SEM of (n) replicates per experimental group. Data were subsequently analyzed by one-way ANOVA, followed by the post hoc Newman-Keuls test for comparisons between group means, using a Prism™ computer program (GraphPad, San Diego, CA, USA). Differences were considered statistically significant at P<0.05.

## Results

**DHA pre-treatment attenuates H<sub>2</sub>O<sub>2</sub>-induced cell death and oxidative damage.** In preliminary experiments, the protective

effects of DHA against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress were investigated in the pheochromocytoma cell line, PC12, by MTS assay. The cells were pre-treated with physiological concentrations of DHA (60  $\mu$ M) for 24 h and then treated with H<sub>2</sub>O<sub>2</sub> for a further 24 h, with the exception of the control groups. A significant and consistent decrease (approximately 40%) in cell viability was observed following 24 h of exposure to H<sub>2</sub>O<sub>2</sub>. Under these conditions, pre-treatment with DHA was able to antagonize the effects of H<sub>2</sub>O<sub>2</sub>. DHA alone did not affect cellular viability in the same experimental paradigm (Fig. 1A).

The cell viability data were also confirmed by subsequent morphological experiments conducted in the same experimental paradigm (Fig. 1B). The number of cells was markedly reduced and many of these had a rounded appearance following exposure to 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Fig. 1B, panel c), compared to the controls and to cells treated with DHA alone (Fig. 1B, panels a and b); pre-treatment with DHA exerted potent protective effects against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage (Fig. 1B, panel D).

A second series of experiments were performed to ascertain whether the protective effects observed post-DHA treatment were associated with the reduction of ROS production and oxidative damage. Under these conditions, pre-treatment (24 h) with DHA was able to reduce ROS production induced by H<sub>2</sub>O<sub>2</sub>, while it was not able to alter the intracellular ROS levels under baseline conditions (Fig. 1C).

Additional experiments were performed to investigate the biochemical mechanisms underlying the antioxidant effects of DHA: Enzymatic (SOD, catalase and GSH-Px) and non-enzymatic (GSH and ascorbic acid) antioxidants, an indicator of lipid peroxidation (MDA) and nitrates and nitrites

Table I. Intracellular levels of enzymatic antioxidants in PC12 cells in both the presence and absence of DHA under different experimental conditions.

Compound	Control mean ± SEM (n)	DHA mean ± SEM (n)	H <sub>2</sub> O <sub>2</sub> mean ± SEM (n)	DHA + H <sub>2</sub> O <sub>2</sub> mean ± SEM (n)
SOD	42.20±2.291 (5)	58.72±2.348 (5) <sup>b</sup>	25.20±2.652 (5) <sup>a</sup>	40.00±6.611 (5) <sup>c</sup>
CAT	23.42±3.149 (5)	19.278±1.332 (5)	10.890±0.599 (5) <sup>b</sup>	12.036±0.579 (5)
GSH-Px	18.36±1.592 (5)	36.55±4.759 (5) <sup>b</sup>	9.15±0.913 (5) <sup>a</sup>	25.69±2.671 (5) <sup>d</sup>

Data are expressed as U/mg protein (see Materials and methods section), and the means ± 1 SEM of 5 replicates per group are shown. <sup>a</sup>P<0.05 and <sup>b</sup>P<0.01 vs. control; <sup>c</sup>P<0.05 and <sup>d</sup>P<0.01 vs. H<sub>2</sub>O<sub>2</sub> alone. SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase.

Table II. Intracellular levels of GSH, ascorbic acid, MDA, nitrate and nitrite in PC12 cells in both the presence and absence of DHA under different experimental conditions.

Compound	Control mean ± SEM (n)	DHA mean ± SEM (n)	H <sub>2</sub> O <sub>2</sub> mean ± SEM (n)	DHA + H <sub>2</sub> O <sub>2</sub> mean ± SEM (n)
GSH	55.25±5.2 (5)	86.65±6.8 (5) <sup>b</sup>	52.47±5.3 (5)	93.11±7.1 (5) <sup>b,d</sup>
Ascorbic acid	2.25±0.52 (5)	2.29±0.7 (5)	0.45±0.09 (5) <sup>a</sup>	0.65±0.1 (5) <sup>a</sup>
MDA	0.01±0.001 (5)	0.02±0.001 (5)	0.03±0.009 (5) <sup>a</sup>	0.01±0.002 (5) <sup>c</sup>
Nitrate	7.45±1.0 (5)	9.81±1.3 (5)	8.89±0.9 (5)	9.48±1.2 (5)
Nitrite	1.28±0.8 (5)	2.00±0.6 (5)	2.91±0.8 (5)	2.15±0.8 (5)

Data are expressed as nmol/mg protein (see Materials and methods section), and the means ± 1 SEM of 5 replicates per group are shown. <sup>a</sup>P<0.05 and <sup>b</sup>P<0.01 vs. control; <sup>c</sup>P<0.05 and <sup>d</sup>P<0.01 vs. H<sub>2</sub>O<sub>2</sub> alone. GSH, reduced glutathione; MDA, malondialdehyde.

were measured in the presence or absence of DHA under basal conditions and in response to oxidative stress. The mean concentration levels of enzymatic antioxidants were markedly lower in the cells exposed to H<sub>2</sub>O<sub>2</sub> than in the controls (Table I). DHA pre-treatment increased the intracellular levels of SOD and GSH-Px in a significant manner under basal conditions and following H<sub>2</sub>O<sub>2</sub>-induced oxidative stress (Table I). The catalase levels were increased by DHA pre-treatment under oxidative stress conditions compared to H<sub>2</sub>O<sub>2</sub> exposure alone, although the difference was not statistically significant. The catalase basal intracellular concentrations remained unaltered (P>0.05) in the presence of DHA (Table I).

In addition, Table II shows the effects of DHA pre-treatment on GSH, ascorbic acid, MDA, nitrates and nitrites under basal and oxidative conditions. It was observed that: i) DHA significantly increased the GSH levels both under baseline conditions and in the presence of H<sub>2</sub>O<sub>2</sub>; ii) DHA increased the intracellular concentrations of ascorbic acid and decreased those of MDA in a statistically significant manner only in the presence of H<sub>2</sub>O<sub>2</sub>; iii) the intracellular levels of nitrates and nitrites remained unaltered following pre-treatment with DHA under both experimental conditions. The results obtained above support the hypothesis that DHA exerts antioxidant effects on H<sub>2</sub>O<sub>2</sub>-exposed PC-12 cells by modulating the levels of antioxidant enzymes and non-enzymatic scavengers, which regulate the levels of ROS.

*DHA pre-treatment attenuates oxidative-stress-induced apoptosis.* PC12 cell morphology was observed by fluorescence microscopy using an Annexin V staining assay to

detect apoptotic cells following pre-treatment with DHA in the presence or absence of oxidative damage. As shown in Fig. 2A, the apoptosis of the cells pre-treated with DHA was visibly decreased compared with that of the cells exposed to H<sub>2</sub>O<sub>2</sub> alone. The process of apoptosis is strongly associated with the loss of ΔΨ<sub>m</sub> in different cells. Indeed, the opening of the mitochondrial permeability transition pore induces the depolarization of the transmembrane potential, and the release of apoptotic factors and the loss of oxidative phosphorylation. Consequently, in this study, in order to determine whether DHA inhibits H<sub>2</sub>O<sub>2</sub>-induced apoptosis related to the mitochondrial pathway, PC12 cells were pre-treated with DHA and exposed to H<sub>2</sub>O<sub>2</sub> to induce mitochondrial dysfunction. The ΔΨ<sub>m</sub> of the cells was reduced with H<sub>2</sub>O<sub>2</sub> alone (60% less than the control), but was enhanced following pre-treatment (24 h) with DHA in a statistically significant manner (Fig. 2B). These results confirmed our hypothesis that DHA is able to inhibit H<sub>2</sub>O<sub>2</sub>-induced mitochondrial dysfunction in PC12 cells.

To confirm this hypothesis further, the subsequent experiments were aimed to investigate the effects of DHA pre-treatment on Bax and Bcl2 gene and protein expression levels. As shown (in Fig. 2C and D, left panel), the expression of the pro-apoptotic gene, Bax, was increased in the H<sub>2</sub>O<sub>2</sub>-exposed cells, while DHA pre-treatment markedly reduced its expression. On the contrary, the expression of anti-apoptotic gene, Bcl2, was decreased following H<sub>2</sub>O<sub>2</sub> exposure, while the gene expression was reversed in the cells pre-treated with DHA (Fig. 2C and D, right panel). The same trend was also observed for the protein levels of Bax and Bcl2 in the same experimental paradigm (Fig. 2E).

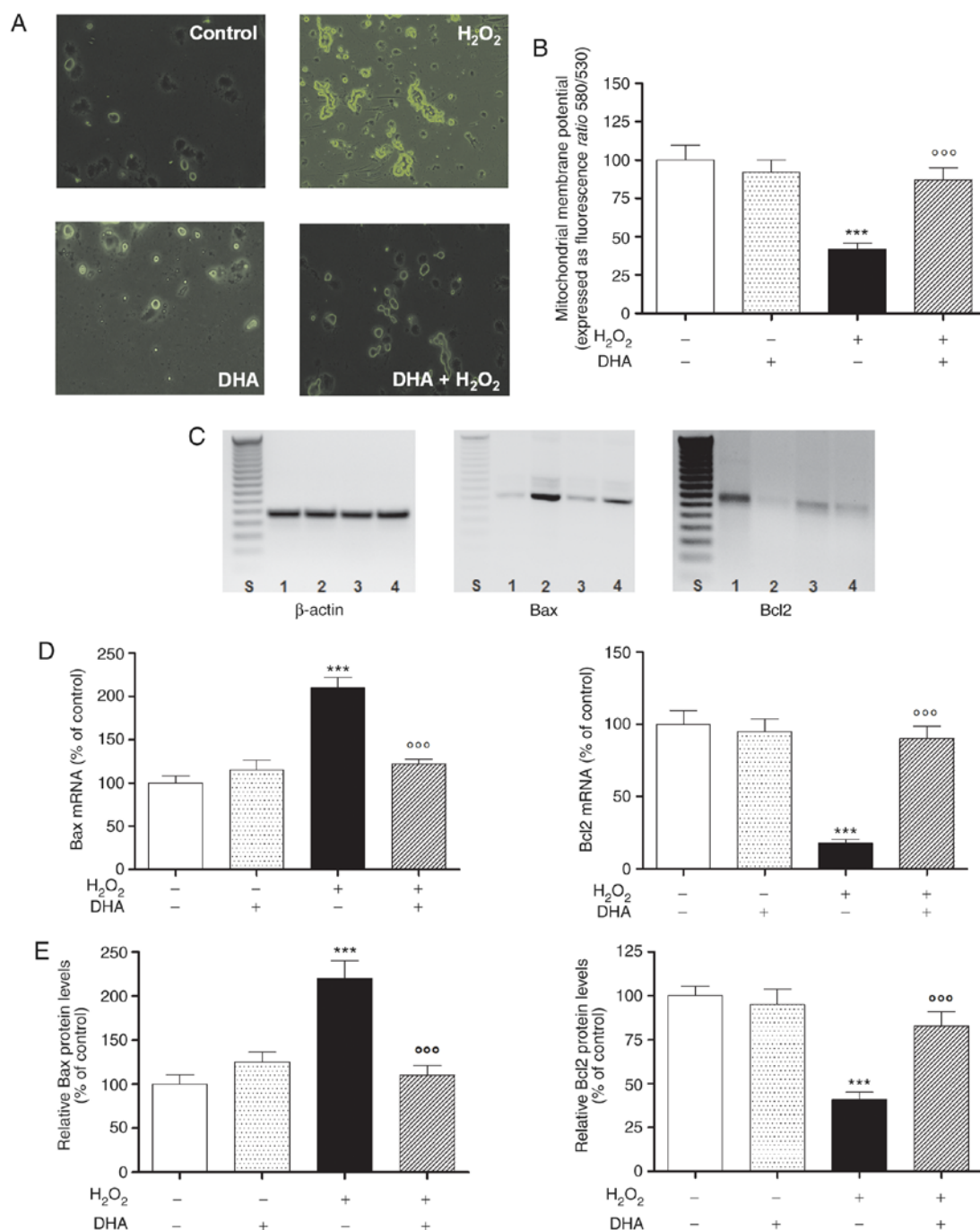


Figure 2. DHA and apoptosis. (A) Fluorescent staining for the detection of the apoptosis of PC12 cells under basal conditions (control) and following treatment with: i) H<sub>2</sub>O<sub>2</sub> (300 μM; 24 h of treatment); ii) DHA (60 μM; 24 h of treatment); iii) DHA plus H<sub>2</sub>O<sub>2</sub> (cells pre-treated for 24 h with DHA and subsequently exposed to H<sub>2</sub>O<sub>2</sub> for a further 24 h). Apoptotic cells were detected by Annexin staining assay. Representative images from 3 independent experiments are shown. (B) The mitochondrial membrane potential ( $\Delta\Psi_m$ ) was measured in PC12 cells by the Mitolight Apoptosis detection kit (see the Materials and methods section for details). The fluorescence intensities were measured at an emission wavelength 580 nm to monitor aggregated molecules (apoptotic cells) and 530 nm for monomer (healthy cells), respectively. Data are expressed as fluorescence intensities ratio (580/530) and reported as a percentage of the control. The means  $\pm$  SEM of 3 determinations in double are shown. (C) Agarose gels representing the mRNA expression levels of  $\beta$ -actin (housekeeping gene, 240 bp), Bax (352 bp) and Bcl2 (349 bp). Lane S, DNA ladder (50 bp); lane 1, untreated cells; lane 2, cells exposed to H<sub>2</sub>O<sub>2</sub> alone; lane 3, cells treated with DHA alone; lane 4, cells pre-conditioned for 24 h with DHA prior to exposure to H<sub>2</sub>O<sub>2</sub> for a further 24 h. (D) Bax (left panel) and Bcl2 (right panel) densitometric analysis of the gels in (C). The density of the gel bands was divided by  $\beta$ -actin and the results were expressed as a percentage of the control band density. Results are from 3 independent experiments performed in duplicate. (E) Effects of pre-treatment with DHA (24 h) on Bax (left panel) and Bcl2 (right panel) protein levels in PC12 cells under baseline conditions and under H<sub>2</sub>O<sub>2</sub> stimulation. Bax and Bcl2 protein levels were detected by ELISA assay and normalized against GADPH (details in Materials and methods section). Results are from 2 independent experiments, each including 3 replicates per experimental group. The data in the figure are presented as the means  $\pm$  SEM. \*\*\* $P$ <0.001 vs. controls; °°° $P$ <0.001 vs. H<sub>2</sub>O<sub>2</sub> alone. DHA, docosahexaenoic acid.

DHA protective effect involves the NFE2L2 antioxidant pathway. To elucidate the plausible signal transduction pathways involved in the DHA-mediated protective effects, we

examined the involvement of NFE2L2, the principal transcription factor that regulates the basal and inducible expression of a number of antioxidant genes. In order to provide direct

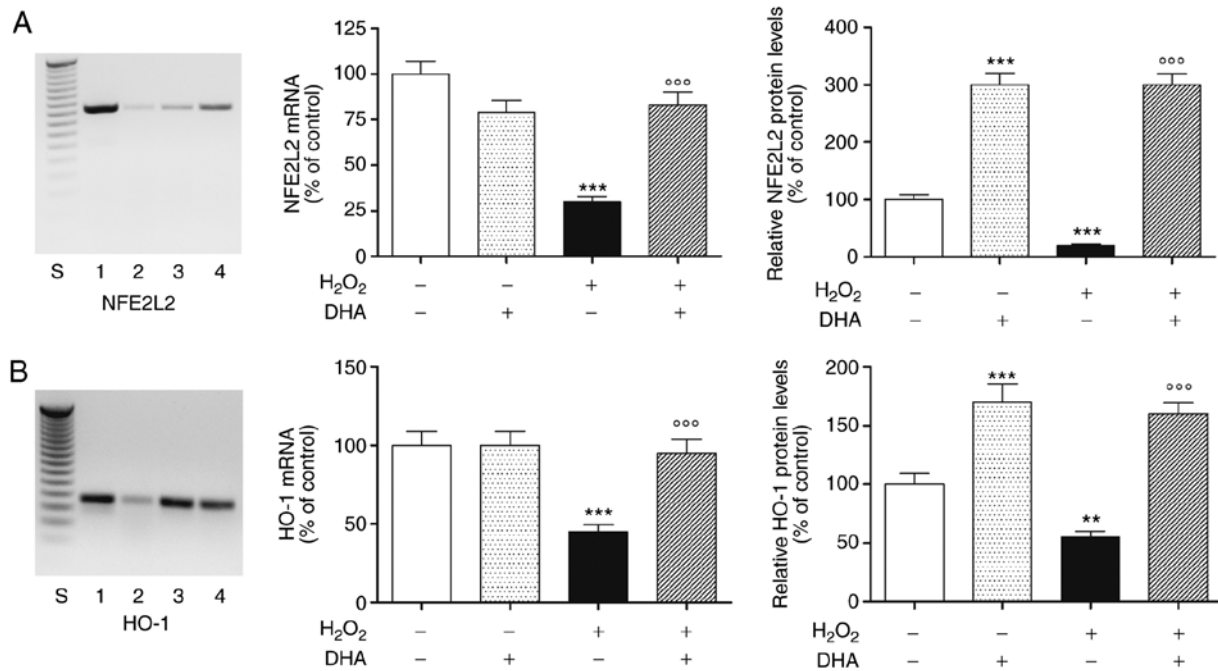


Figure 3. Agarose gels representing (A) the mRNA expression levels of NFE2L2 (466 bp) and (B) HO-1 (225 bp) in PC12 cells. Lane S, DNA ladder (50 bp); lane 1, untreated cells; lane 2, cells exposed to H<sub>2</sub>O<sub>2</sub> alone; lane 3, cells treated with DHA alone; lane 4, cells pre-conditioned for 24 h with DHA prior to exposure to H<sub>2</sub>O<sub>2</sub> for a further 24 h. The density of the gel bands was divided by that of β-actin (Fig. 2) and expressed as a percentage of the control band density. All results are from 3 independent experiments. Data are presented as the means ± SEM. \*\*P<0.01 and \*\*\*P<0.001 vs. control; °°°P<0.001 vs. H<sub>2</sub>O<sub>2</sub> alone. DHA, docosahexaenoic acid; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme-oxygenase-1.

evidence of the involvement of NFE2L2 activation, the PC12 cells were pre-treated with DHA for 24 h, followed by exposure to H<sub>2</sub>O<sub>2</sub> for an additional 24 h. We observed in our experimental paradigm that H<sub>2</sub>O<sub>2</sub> markedly downregulated NFE2L2 mRNA expression, which was antagonized by pre-treatment with DHA (Fig. 3A, left and middle panels). Notably, DHA was effective only in the presence of oxidative damage; in fact, pre-treatment with DHA alone did not stimulate NFE2L2 gene expression, but rather decreased it (Fig. 3A, left and middle panels). The results obtained were also confirmed by subsequent experiments conducted to determine the NFE2L2 protein intracellular levels. In particular, the NFE2L2 protein levels increased >3-fold following DHA pre-treatment both under basal conditions and following H<sub>2</sub>O<sub>2</sub>-induced oxidative damage (Fig. 3A, right panel).

The transcriptional activation of NFE2L2 evokes the activation of a series of phase II detoxifying enzymes, including HO-1, which is able to protect cells against oxidative damage. Therefore, to further investigate whether DHA-induced NFE2L2 activation modulate in turn HO-1, we examined the effects of DHA pre-treatment on the activation of the NFE2L2/HO-1 pathway in PC12 cells in same experimental paradigm. The intracellular mRNA expression and protein levels of HO-1 were markedly decreased to an estimated 50% following exposure to H<sub>2</sub>O<sub>2</sub> in comparison to the untreated cells. The observed effect was markedly inhibited by DHA pre-treatment (24 h), which increased the mRNA expression and protein levels of HO-1 to values equal or higher than those observed in the untreated cells, respectively (Fig. 3; pan B). Under baseline conditions, the DHA alone significantly increased the HO-1 protein levels, while it had no effect on the mRNA expression of the enzyme (Fig. 3B).

## Discussion

The wide variety of physiopathological relapses, generated in the organism by redox imbalance, can explain why oxidative stress has been implicated in the pathogenesis and complications of a number of neurodegenerative diseases. Therefore, evaluating oxidative stress and ROS (which are considered to be mediators of oxidative damage) levels and attempting to counteract the progression of damage, can be limited. A number of edible natural compounds have been shown to modulate ROS production and consequently, oxidative damage. DHA, a dietary fat, is a representative n-3 PUFA, which has a wide array of health beneficial effects; of note are its effects at the central nervous system level (21-23), as it has been shown to decrease the risk of developing Alzheimer's disease (24). Although the protective effects of DHA have long been associated with the modification of cell membrane fluidity, there is new evidence to suggest that the action of this fatty acid may be attributed to the modulation of anti-inflammatory gene expression and anti-oxidative pathways (25,26). However, the biochemical mechanisms underlying the protective effects of DHA remain unclear. Thus, in the present study, we focused on investigating the potential protective effects of DHA at physiological concentrations against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in rat pheochromocytoma cells (PC12), exploring cell viability, the oxidation potential, and the anti-apoptotic and antioxidant effects.

H<sub>2</sub>O<sub>2</sub> is currently the most widely used experimental model for simulating oxidative stress. In fact, H<sub>2</sub>O<sub>2</sub> belongs to ROS and is potentially harmful to cell viability as it damages different cellular targets. As expected, H<sub>2</sub>O<sub>2</sub> was found to be toxic to cells in our experimental model, leading to a marked decrease in the overall cellular viability, an increase in ROS

levels and to the activation of the apoptotic cascade. These effects were antagonized by treatment with DHA for 24 h prior to exposure to H<sub>2</sub>O<sub>2</sub>. Pre-treatment with DHA increased the antioxidant activity by an increase in cell viability and a reduction in ROS levels, thus protecting the cells against oxidative stress-induced cell damage. The cells have integrated antioxidant systems, which include enzymatic and non-enzymatic antioxidants, that are usually effective in blocking the noxious effects of ROS (27). However, under pathological conditions, these systems may be insufficient (28). In this study, we demonstrated that DHA was able to reverse the H<sub>2</sub>O<sub>2</sub>-induced intracellular levels of a few of these antioxidants (SOD, catalase, GSH-Px, GSH, ascorbic acid and MDA). Furthermore, we observed a clear reduction in the apoptotic signals by DHA, which exerted both a protective effect at the mitochondrial level and played a regulatory role in the modulation of Bax- and Bcl2-mediated apoptosis, both negative and positive. Overall, these findings suggest that DHA may be able to protect the cells against stress-induced apoptosis, mainly via the protection of the mitochondrial pathway.

Notably, it has been recently identified that NFE2L2 is able to activate antioxidant cellular defences and to inhibit apoptotic pathways (29). NFE2L2 is a regulator of cellular resistance against oxidants. It controls the gene expression of a large variety of antioxidant/cytoprotective enzymes and detoxification phase II enzymes, regulating the physiological and pathophysiological outcomes of oxidant exposure. In fact, oxidative stress dissociates NFE2L2 from its cytoplasmic inhibitor, Kelch-like ECH-associated protein 1 (KEAP1), promoting its translocation into the nucleus, where it binds to AREs leading to the transcriptional activation of antioxidant/cytoprotective enzymes, such as HO-1, SOD and subunits which are necessary for GSH biosynthesis (30). In particular, HO-1 antagonizes oxidative stress by activating the expression of SOD (31) and through the degradation of the pro-oxidant, heme, leading to the production of bilirubin and CO, that are important antioxidants (32-34). Moreover, under stress conditions, NFE2L2 directly prevents apoptosis by activating Bcl-2, inhibiting Bax and through the activation of p62, and reduces mitochondrial dysfunctions. The findings obtained in this study coincide with the aforementioned: We observed a significant increase in the cellular levels of NFE2L2 following pre-incubation with DHA in our experimental model, and we thus hypothesized that this PUFA can increase the levels of key antioxidant enzymes, such as SOD, GSH-Px and HO-1, as well as modulate apoptotic pathways.

In this study, we measured the level of NFE2L2 in the whole cell, based on previously published data (35,36) demonstrating that when PC12 cells are subjected to oxidative stress, NFE2L2 is exclusively present at nuclear level. However the observed mechanism may be associated mainly with a direct action of DHA on availability of NFE2L2 in monomeric form, rather than on gene expression, as evidenced by the data on gene expression and protein levels of NEF2L2. In fact, the mRNA expression levels of both NFE2L2 and HO-1 remained almost similar to the controls following pre-treatment with DHA, in the presence of H<sub>2</sub>O<sub>2</sub> but, above all, under basal conditions. This direct action at the post-transcriptional level could also explain the directness with which DHA, after 24 h of pre-treatment, counteracted the oxidizing and apoptotic

effects exerted by H<sub>2</sub>O<sub>2</sub>. Notably, while the exposure of cells to H<sub>2</sub>O<sub>2</sub> is a well-established experimental model of oxidative damage, the effects of H<sub>2</sub>O<sub>2</sub> on NEF2L2 activation and the expression of its downstream gene products remains controversial and unclear. In fact, on the one hand, it has been shown that H<sub>2</sub>O<sub>2</sub> stabilizes and activates NEF2L2 by the oxidation and de-activation of KEAP1 (35), while on the other hand, it has been observed that exposure to H<sub>2</sub>O<sub>2</sub> reduces the mRNA and protein levels of NEF2L2 (35-41), hypothesizing an involvement of NF-κB (a negative regulator of the expression of NEF2L2) (38), or rather an upregulation of KEAP1 during oxidative stress, thus leading to the inhibition of NEF2L2 (40,41). Consistent with literature data on PC12 cells (35,36), we observed in our experimental paradigm, that H<sub>2</sub>O<sub>2</sub> markedly downregulated NFE2L2 expression, which was antagonized by pre-treatment with DHA.

In conclusion, in this study, we demonstrate that DHA is able to protect PC12 cells from H<sub>2</sub>O<sub>2</sub>-induced oxidative damage through a mechanism involving the activation of the NFE2L2/HO-1 signaling pathway. These findings suggest that a strong link could exist between an adequate supply in the diet of this essential PUFA and the maintenance of the antioxidant potential of the neuronal cells. Thus, in light of these findings, it can be hypothesized that DHA may prove to be a potential therapeutic agent for the prevention of diseases characterized by inflammation and oxidative stress at the central nervous system level.

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#### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

#### Authors' contributions

MEC, GL and GT conceived and designed the study, performed the experiments, contributed to data analysis and wrote the manuscript. GL reviewed and supported MEC and GT in revisions of the manuscript. BS and AB performed the experiments, conceptualized the study design, and contributed to data analysis and experimental materials. All authors have read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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