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Nonenzymatic oxygenated metabolites of α-linolenic acid B_1- and L_1-phytoprostanes protect immature neurons from oxidant injury and promote differentiation of oligodendrocyte progenitors through PPAR-γ activation

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A B S T R A C T

Phytoprostanes (PhytoP's) are formed in higher plants from α-linolenic acid via a nonenzymatic free radical-catalyzed pathway and act as endogenous mediators capable of protecting cells from damage under various conditions related to oxidative stress. Humans are exposed to PhytoP's, as they are present in relevant quantities in vegetable food and pollen. The uptake of PhytoP's through the olfactory epithelium of the nasal mucosa, upon pollen grain inhalation, is of interest as the intranasal pathway is regarded as a direct route of communication between the environment and the brain. On this basis, we sought to investigate the potential activities of PhytoP's on immature cells of the central nervous system, which are particularly susceptible to oxidative stress. In neuroblastoma SH-SY5Y cells, used as a model for undifferentiated neurons, B_1-PhytoP's, but not F_1-PhytoP's, increased cell metabolic activity and protected them from oxidant damage caused by H_2O_2. Moreover, B_1-PhytoP's induced a moderate depolarization of the mitochondrial inner membrane potential. These effects were prevented by the PPAR-γ antagonist GW9662. When SH-SY5Y cells were induced to differentiate toward a more mature phenotype, they became resistant to B_1-PhytoP activities. B_1-PhytoP's also influenced immature cells of an oligodendroglial line, as they increased the metabolic activity of oligodendrocyte progenitors and strongly accelerated their differentiation to immature oligodendrocytes, through mechanisms at least partially dependent on PPAR-γ activity. However, B_1-PhytoP's did not protect oligodendrocyte progenitors against oxidant injury. Taken together, these data suggest that B_1-PhytoP's, through novel mechanisms involving PPAR-γ, can specifically affect immature brain cells, such as neuroblasts and oligodendrocyte progenitors, thereby conferring neuroprotection against oxidant injury and promoting myelination.

Free radical-catalyzed oxidation of polyunsaturated fatty acids (PUFAs)[2] is a hallmark of oxidative stress. Arachidonic acid (AA; C20:4ω6) and docosahexaenoic acid (DHA; C22:6ω3) are the most abundant PUFAs in mammals, and their peroxidated products—isoprostanes (IsoP's) and neuroprostanes (NeuroP's), respectively—are considered among the most sensitive and reliable biomarkers of oxidative stress [1,2]. IsoP's and NeuroP's are large families of regio- and stereoisomeric prostaglandin-like molecules; some of them have been shown to display significant biological activities that could contribute to as well as protect from oxidant injury.

A third biologically active lipid family is represented by phytoprostanes (PhytoP's), which are formed in plants via a nonenzymatic

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; bFGF, basic fibroblast growth factor; CV, crystal violet; DHA, docosahexaenoic acid; IsoP, isoprostane; MAP2, microtubule-associated protein 2; mMP, mitochondrial inner membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NeuroP, neuroprostane; OL, oligodendrocyte; OP, oligodendrocyte progenitor; PDGF, platelet growth factor; PhytoP, phytoprostane; PPAR-γ, peroxisome proliferator-activated receptor γ; PUFA, polyunsaturated fatty acid; RA, retinoic acid; TMRE, tetramethylrhodamine ethyl ester perchlorate

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free radical-catalyzed pathway analogous to that leading to IsoP and NeuroP formation [3]. The PhytoP precursor is α-linolenic acid (ALA; C18:3ω3), a predominant PUFA in higher plants, generally lacking the enzymatic capacity to form longer chain PUFAs such as AA and DHA. Several classes of PhytoP's are constitutively present in plants and their levels rise in response to oxidative stress [4]. As their counterparts in the animal kingdom, PhytoP's occur in several classes, named according the prostaglandin classification, each of which can be generated as two racemic regioisomers (for further details see [5] and Fig. 1). The development of a new chemical strategy, based on a furan approach, has led to the synthesis of enantiomerically pure B_1-, F_1-, and E_1-PhytoP's [6,7], thus allowing the full assessment of the biological activities of each of these compounds.

Among the best studied PhytoP's are those of the A and B classes. Several lines of evidence indicate that A_1- and B_1-PhytoP's in plant cells influence the expression of numerous genes, most of which are involved in the detoxification of xenobiotics and in cytoprotective responses, suggesting that B_1-PhytoP's represent endogenous mediators capable of counteracting cell damage (see [8] and references therein). Much less is known of the potential biological activities of PhytoP's in mammalian systems.

Humans are potentially exposed to PhytoP's. Vegetable foods, in particular vegetable oils, contain high levels of ALA, which could be converted into PhytoP's by autoxidation during cooking and/or storage or after oral consumption in the gastrointestinal tract. Karg et al. [9] have shown that PhytoP's of the A_1, B_1, E_1, and F_1 classes are present in up to milligram quantities per 100 ml in fresh vegetable oil, with the highest levels found in linseed and soybean oils. These levels may further increase by more than an order of magnitude with storage, reaching submillimolar concentrations. After oral ingestion, PhytoP's are adsorbed and found in plasma and urine in conjugate and free form, respectively. Plasma and urinary levels of F_1-PhytoP's were found increased in healthy men after 4 weeks of flaxseed oil supplementation compared to a control group receiving olive oil supplementation [10]. PhytoP's have also been detected in parenteral lipid nutrition used in intensive care medicine, containing lipid fractions from vegetable oils such as soybean and olive oils [10].

In addition to oral ingestion, humans can be exposed to PhytoP's through inhalation of pollen, rich in both ALA and PhytoP's. Upon contact with the respiratory mucosa, pollen grains release allergens in conjunction with many other substances, including bioactive lipids, collectively called pollen-associated lipid mediators. Aqueous pollen extracts contain significant levels of E_1-, F_1-, A_1-, and B_1-PhytoP's. Of these, E_1-PhytoP is among the most prominent and it has been identified as one of the pollen-associated lipid mediators, capable of modulating dendritic cell functions and favoring a type 2-dominated proallergenic immune response [11,12]. The fact that PhytoP's can be released from pollen grains upon contact with the airway mucosa, and in particular nasal mucosa, is intriguing as the intranasal pathway is regarded as a direct route of communication between the environment and the brain.

In addition to the above-mentioned immunomodulatory activity of E_1-PhytoP on human dendritic cells, few other PhytoP biological

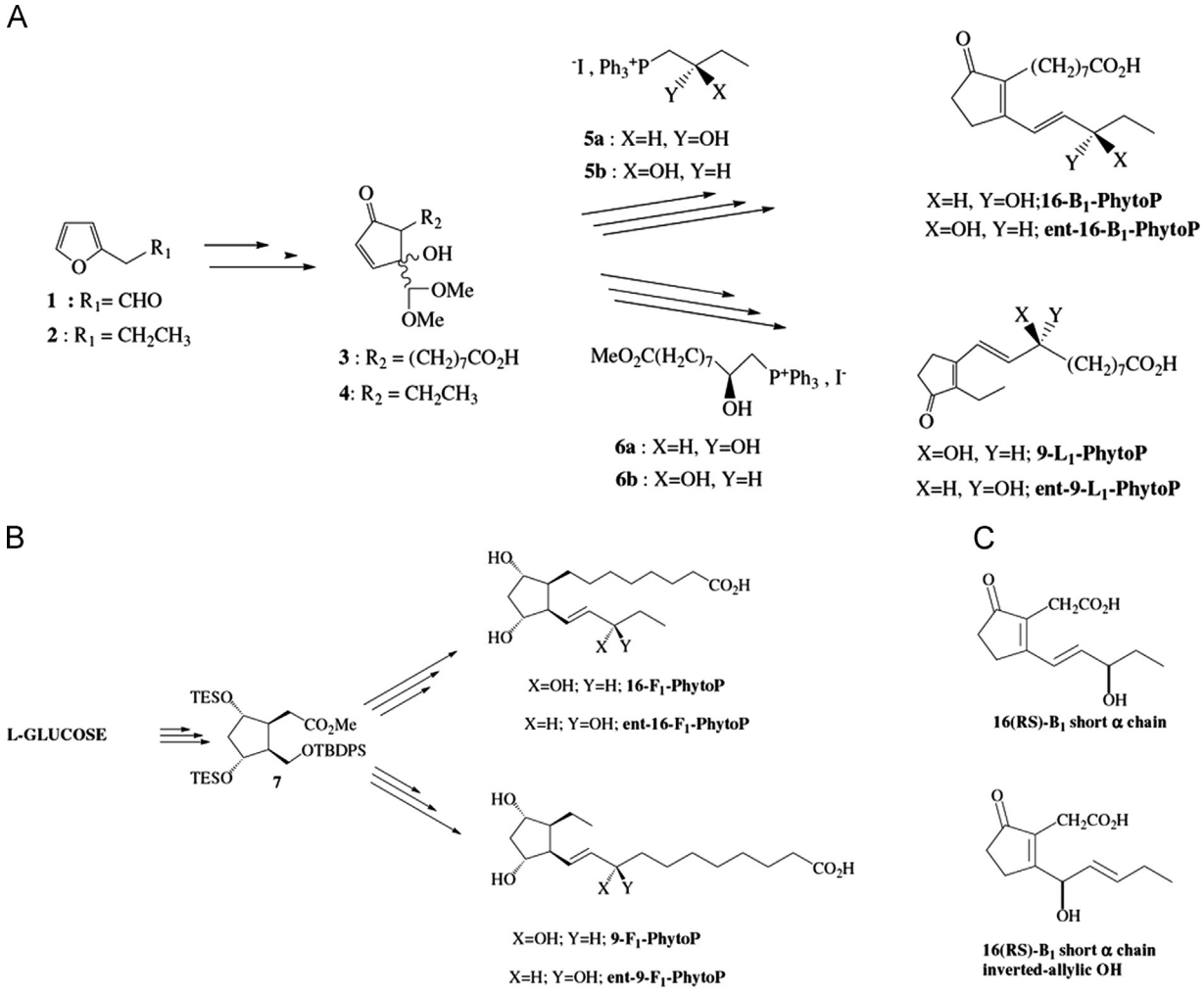

Fig. 1. Total synthesis of F_1-, B_1-, and L_1-PhytoP's and analogs. See Materials and methods for details.

activities on animal systems have been reported. A_1- and B_1-PhytoP's have been shown to inhibit the release of nitric oxide in lipopolysaccharide-stimulated RAW264.7 macrophages, whereas in leukemia Jurkat T cells A_1- but not B_1-PhytoP's trigger apoptosis [9].

Taken together, these observations suggest that PhytoP's are bioactive lipids in both plant and animal systems and highlight the importance of better understanding their potential biological roles, in particular with respect to oxidative stress [11].

On this basis we sought to investigate the possible effects of B_1- and F_1-PhytoP's on immature cells of the central nervous system, which are particularly susceptible to oxidative stress. As experimental models we used SH-SY5Y cells, a cell line derived from a human neuroblastoma and resembling neuroblasts [13], and primary cultures of rat oligodendrocyte (OL) progenitors (OPs) [14]. To induce oxidant injury, cells were exposed to hydrogen peroxide (H_2O_2). We show that B_1-PhytoP's protect SH-SY5Y cells against oxidant injury through mechanisms involving activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR)-γ. In these cells, B_1-PhytoP's induce a moderate depolarization of the mitochondrial inner membrane potential (mMP), which is also dependent on PPAR-γ activation and could be part of the defensive response triggered by B_1-PhytoP's. Upon differentiation, SH-SY5Y cells become resistant to B_1-PhytoP activities. Similarly, B_1-PhytoP's do not protect OPs against oxidant injury but they strongly accelerate OP differentiation to immature OLs. Again, B_1-PhytoP-induced OL differentiation was at least partially dependent on PPAR-γ activity.

Materials and methods

Synthesis of phytoprostanes

The two regioisomers 16-B_1- and 9-L_1-PhytoP, previously known as PPB_1 type I and type II (Fig. 1A), were prepared by total synthesis according to our published procedures [15]. Briefly, the 3-hydroxy-4-cyclopentenone key intermediates (3 and 4, Fig. 1A) in our furan strategies were obtained from the commercially available furfural and *n*-propylfuran (1 and 2, Fig. 1A) in seven and five steps, respectively, with good yields. The introduction of the α and ω chains of PhytoP's bearing the hydroxyl allylic chiral center was achieved using chiral phosphonium salts (5 and 6, Fig. 1A). The synthesis of the 16-F_1- and 9-F_1-PhytoP's (Fig. 1B) was achieved using our earlier strategy based on a radical cyclization step, starting from commercially available L-glucose as chiral pools [6].

Cell cultures

Human SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA, USA) were cultured in 1/1 Dulbecco's modified Eagle's medium (DMEM; Lonza, Milan, Italy)/Ham's F12 medium (Cambrex Bio Science–Verviers, Verviers, Belgium) supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and antibiotics 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Eugene, OR, USA). Cell cultures were maintained in a 5.0% CO_2 humidified atmosphere at 37 °C and split at about 80% confluence for a maximum of 20 passages. Cells were harvested by trypsinization and the cell suspension was resuspended in fresh medium and seeded at a density of 10^5 cells/cm^2 in 96-well plates (BD Falcon, Franklin Lakes, NJ, USA), for 3-(4,5-dimethyl thiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) or crystal violet (CV) assay, or 6-well plates for Western blot or plated on glass coverslips (Steroglass, Perugia, Italy) for video imaging or immunocytochemistry experiments. Cell differentiation was induced by exposing SH-SY5Y cells, after 24 h of plating, to 5 μM retinoic acid (RA; Sigma, St. Louis, MO, USA) in complete growth medium. The cells were maintained under these conditions for 5 days with medium change every 2 days.

Purified cultures of OPs were prepared from newborn Wistar rats as described [14] and in accordance with the European Communities Council Directive No. 86/609/EEC. OPs growing on top of mixed glial monolayers were mechanically detached and seeded at the density of 6×10^4 cells/cm^2 into poly-L-lysine-coated 35-mm-diameter plastic culture dishes or 96-well plates. At 2 h after plating, the culture medium (DMEM with high glucose, supplemented with 10% fetal calf serum) was replaced with a chemically defined serum-free medium, consisting of DMEM/Ham's F12 (4/1) supplemented with 5.6 mg/ml glucose, 5 μg/ml insulin, 100 μg/ml human transferrin, 100 μg/ml bovine serum albumin, 0.06 ng/ml progesterone, 40 ng/ml sodium selenite, 16 μg/ml putrescine, bFGF, PDGF, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. All supplements were from Sigma. After 24 h, the cells were exposed to several conditions as detailed in the figure legends.

MTT and crystal violet assays

The ability of cells to reduce MTT (Sigma) was assessed as an index of cellular metabolic activity and mitochondrial integrity, as previously described [14]. MTT was added at a final concentration of 0.25 mg/ml during the final 4 h of incubation. The medium was then removed, and 100 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the dark blue crystals. The plates were then read on a microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The cell number in each condition was estimated by CV dye [18]. Protein concentration was measured by BCA protein assay (Thermo/Pierce, Rockford, IL, USA).

Determination of mitochondrial membrane potential

To measure mitochondrial membrane potential we used the potentiometric dye tetramethylrhodamine ethyl ester perchlorate (TMRE; Sigma) at a final concentration of 30 nM (from 1 mM stock solution in DMSO). Cells were kept for 30 min (OPs) or 50 min (SH-SY5Y cells) in the presence of TMRE before recording, to reach saturation of the dye, and maintained throughout the entire experiment to avoid the decay of the signal. An oil immersion objective (Olympus; 40 ×, 1.35 NA) mounted on an inverted microscope (Axiovert 135; Carl Zeiss, Oberkochen Germany) was utilized for fluorescence video imaging. The excitation wavelength 535 nm was applied by means of a monochromator (Till Photonics, Polychrome II, Munich, Germany) and the emission light at 590 was collected by a CCD, cooled digital camera (PCO, Sensicam, Kelheim, Germany) and recorded. The Imaging Workbench 6.0 software package (Indec BioSystems, Santa Clara, CA, USA) was used for recording and offline analysis of the data. The software allowed the measurement of the emission value in regions of interest or along line profiles, where peaks of amplitude corresponded to single mitochondria. The average amplitudes of the fluorescence intensity were calculated (from a minimum of three experiments for each condition) and shown in bar graphs as the average ± SEM, with *n* representing the number of observations.

Immunofluorescence

SH-SY5Y cells were fixed in 4% paraformaldehyde and permeabilized (0.2% Triton X-100/phosphate-buffered saline (PBS)). After a blocking step in 3% bovine serum albumin (BSA) Triton X-100/PBS, the cells were incubated with rabbit anti-MAP2 polyclonal antibodies (1:500 in 3% BSA/PBS) 2 h at room temperature. After

being rinsed, the cells were incubated with Cy3 donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:200) in PBS, 1 h at room temperature. Nuclei were stained using Hoechst 33258 (5 μg/ml for 20 min, Sigma, Italy). Coverslips were then mounted with DAKO fluorescence mounting medium.

OPs were incubated with the oligodendroglial marker O_4, by using a primary monoclonal antibody (mouse immunoglobulin M (IgM), hybridoma supernatants; diluted 1:5) and, as secondary antibody, fluorescein-conjugated goat anti-mouse IgM (1:200, Jackson ImmunoResearch Laboratories), before fixation in 4% paraformaldehyde. For PPAR-γ translocation, cells were incubated overnight at 4 °C with anti-PPAR-γ polyclonal rabbit antibodies (1:100; Calbiochem, San Diego, CA, USA) and processed with secondary antibodies as before. Coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and examined using a Leica DM4000B fluorescence microscope equipped with a DFC420C digital camera and Leica Application Suite software (260RI) for image acquisition. Cells were counted in 10 microscopic fields of 0.18 mm^2 per coverslip prepared in duplicate for each condition from at least three independent experiments.

Statistical analysis

Data are expressed as means ± SEM of n independent experiments (run in duplicate). Statistical significance was evaluated using Student's t test or one-way ANOVA for multiple comparisons. Numbers of independent experiments are indicated in the figure legends; $p < 0.05$ was accepted as statistical significance. Analyses were performed using Stata 8.1 software (Stata Corp., College Station, TX, USA).

Results

B_1- but not F_1-phytoprostanes enhance SH-SY5Y cell metabolic activity

The human neuroblastoma SH-SY5Y cell line is widely used as a model cell system for studying neuronal death induced by oxidative stress and, in particular, H_2O_2-induced oxidant injury [16]. We first examined the effects of 16-B_1-PhytoP, 9-L_1-PytoP, and 9- and 16-F_1-PytoP's (Fig. 1A and B) on cell viability. SH-SY5Y cells were exposed for 24 h to the various PhytoP's (0.1–10 μM), and cell viability was examined by MTT reduction assay. 16-B_1-PhytoP and 9-L_1-PhytoP increased the ability of SH-SY5Y cells to reduce MTT, whereas 16-F_1-PhytoP and 9-F_1-PhytoP had no effect (Fig. 2A), suggesting that the B_1 cyclopentenone ring, but not the F_1 cyclopentane ring, is important for PhytoP activity on SH-SY5Y cells. On this basis we focused our study on the B_1 series.

MTT reduction assay is based on the ability of living cells to take up MTT and to reduce it by NADH-dependent dehydrogenases and it is generally used as a measure of cell viability. To verify whether the B_1-PhytoP-induced increase in MTT reduction was due to an enhanced metabolic activity or, rather, to cell proliferation, we evaluated the cell number using CV, a dye that binds to DNA. As shown in Fig. 2B, there was no difference between 16-B_1-PhytoP- and 9-L_1-PhytoP-treated and untreated cultures, suggesting that both 16-B_1-PhytoP and 9-L_1-PhytoP types increase cell metabolic activity rather than cell proliferation.

As opposed to 16-B_1-PhytoP, the cyclopentenone isoprostane (15-A_{2t}-IsoP), an oxidized arachidonic acid metabolite, induced a significant cell impairment, when tested at comparable concentration range (Fig. 2C), as previously described in primary hippocampal neurons [17].

To gain more information on the relation between biological activity and chemical structure, we tested the 16-B_1-PhytoP and 9-L_1-PhytoP enantiomers (Fig. 1A) and two B_1-PhytoP derivatives characterized by a short side chain and alternative allylic alcohol migration, as depicted in Fig. 1C. Of the four compounds, only *ent*-16-B_1-PhytoP induced a significant increase in MTT reduction, when tested at high concentration (25 μM, Fig. 3A). Altogether, these results suggest that allylic configuration and the position of the carboxyl group with respect to the cyclopentenone ring are two important factors controlling the B_1-PhytoP biological profile.

16-B_1-PhytoP and 9-L_1-PhytoP protect undifferentiated SH-SY5Y cells against oxidant injury

We next examined whether PhytoP's could protect SH-SY5Y cells against H_2O_2-induced oxidant injury. Cells were exposed to increasing concentrations of H_2O_2 to identify the concentration yielding an approximately 50% cell impairment, as assessed by MTT reduction assay (Fig. 4A). On the basis of this dose–response curve, cells were then exposed to 0.25 mM H_2O_2 alone or in combination with 16-B_1-PhytoP, 9-L_1-PhytoP, or their enantiomers, at two concentrations (10 and 25 μM). Both 16-B_1-PhytoP and 9-L_1-PhytoP prevented the H_2O_2-dependent cell impairment. As before, of the two enantiomers, only *ent*-16-B_1-PhytoP showed a protective activity, at both concentrations tested (Fig. 4B).

Undifferentiated SH-SY5Y cells are neuroblast-like cells that can acquire a functional neuron-like phenotype in response to treatment with various agents, including RA. Because differentiation is known to be associated with changes in susceptibility to both neurotoxins and neuroprotective substances [18], we tested the biological activity of 16-B_1-PhytoP and 9-L_1-PhytoP on SH-SY5Y cells after differentiation. To this aim, SH-SY5Y cells were exposed to 5 μM RA added to the culture medium at days 1 and 3, according to Miglio et al. [19]. At day 5, compared to control cultures, RA-treated cells showed a neuron-like morphology and higher expression of microtubule-associated protein 2 (MAP2), a protein associated with microtubules of differentiated neurons (Fig. 5A). Differentiated SH-SY5Y cells were exposed to H_2O_2 and or PhytoP's at day 4 for 24 h. As shown in Fig. 5C, differentiated SH-SY5Y cells were less sensitive to H_2O_2-induced oxidant injury compared to the undifferentiated cells, and 0.5 mM concentration was required to obtain an approximately 40% impairment. When differentiated cells were challenged with 16-B_1-PhytoP, 9-L_1-PhytoP, or the inactive *ent*-9-L_1-PhytoP, alone or in combination with 0.5 mM H_2O_2 (Fig. 5D), none of the three compounds affected MTT reduction by themselves or rescued cells from H_2O_2 toxicity, suggesting that the biological activities of B_1-PhytoP's on neuronal cells are dependent on their differentiation state.

16-B_1-PhytoP protects undifferentiated SH-SY5Y cells through PPAR-γ-mediated mechanisms

A mechanism of action proposed for PhytoP's, in particular E_1-PhytoP's, is the activation of the nuclear receptor PPAR-γ. To assess whether this receptor was mediating B_1-PhytoP protective activity on undifferentiated SH-SY5Y cells, we focused on 16-B_1-PhytoP. SH-SY5Y cells were challenged with H_2O_2 and 16-B_1-PhytoP, alone or in the presence of the specific PPAR-γ antagonist GW9662 (Fig. 6A). The antagonist did not modify per se the toxic activity of H_2O_2 but it prevented the protective effect of 16-B_1-PhytoP, indicating that PPAR-γ activation is necessary for the protection against H_2O_2-induced oxidant injury.

Several lines of evidence indicate that in plants 16-B_1-PhytoP and 9-L_1-PhytoP may influence the expression of adaptive-response genes, enabling cells to counteract cell damage caused by various toxicants and, in particular, those causing oxidative stress. PPAR-γ is a ligand-dependent transcription factor regulating the expression of several target genes, including antioxidant enzymes such as catalase and Cu/Zn-superoxide dismutase. However, we did not observe any

effect of 16-B_1-PhytoP expression on these enzymes after 24 h of treatment (not shown).

A potentially important mechanism for promoting cellular protection against oxidant stress is a slight degree of depolarization of the inner membrane of mitochondria [20]. To examine if this was the case in 16-B_1-PhytoP-treated SH-SY5Y cells, we used the potentiometric dye TMRE in single-cell video-imaging experiments to measure the changes in mMP in cells exposed for 24 h to either 16-B_1-PhytoP or the inactive *ent*-9-L_1-PhytoP. The intensities of the TMRE fluorescence signal were decreased only in cells treated with 16-B_1-PhytoP (Fig. 6B). As for the protective effect against H_2O_2 toxicity, the presence of the PPAR-γ antagonist GW9662 abolished the decrease in mMP caused by 16-B_1-PhytoP, without affecting the mMP of untreated cells (Fig. 6C).

16-B_1-PhytoP and 9-L_1-PhytoP do not protect oligodendrocyte progenitors but promote their differentiation

OPs are highly susceptible to oxidant injury and represent a target of oxidative stress in the brain. OPs, isolated from mixed glial cultures, were grown in chemically defined medium in the presence of growth factors (bFGF and PDGF) for 24 h before addition of 16-B_1-, 9-L_1-, or *ent*-9-L_1-PhytoP's. Cell viability was tested after 24 h (2 days in vitro cultures, 2 DIV) by MTT assay. Of the three compounds, only 16-B_1-PhytoP significantly increased the ability of OPs to reduce MTT (Fig. 7A). However, in the presence of 0.025 or 0.05 mM H_2O_2 (giving a reduction of about 20 and 50% of cell viability, respectively), 16-B_1-PhytoP, tested at two concentrations (1 and 10 μM), was unable to prevent or mitigate cell impairment (Fig. 7B). Interestingly, the 24-h exposure of OPs to 1 μM 16-B_1-PhytoP, but not to *ent*-9-L_1-PhytoP, promoted OP maturation as indicated by the increased number of cells expressing O_4, a marker for immature OLs (Fig. 8A). In addition, compared to control cultures, 16-B_1-PhytoP-treated cultures exhibited a more complex and branched morphology, another hallmark of oligodendrocyte differentiation (Fig. 7B). The effect on morphology was more evident after 72 h of exposure to 16-B_1-PhytoP (Fig. 8B, 4 DIV).

Neither 16-B_1-PhytoP nor *ent*-9-L_1-PhytoP significantly affected mitochondrial membrane potential in OP cultures exposed for 24 h to the compounds (Fig. 8C). However, as in the case of protection in SH-SY5Y cells, the effect of 16-B_1-PhytoP in OP cultures was at least in part due to PPAR-γ activation. As shown in Fig. 8D, the presence of the specific PPAR-γ antagonist GW9662 partially prevented the 16-B_1-PhytoP-dependent increase in the percentage of O_4-immunopositive cells. To further support the involvement of PPAR-γ, we evaluated the nuclear translocation of the receptor known to be induced upon

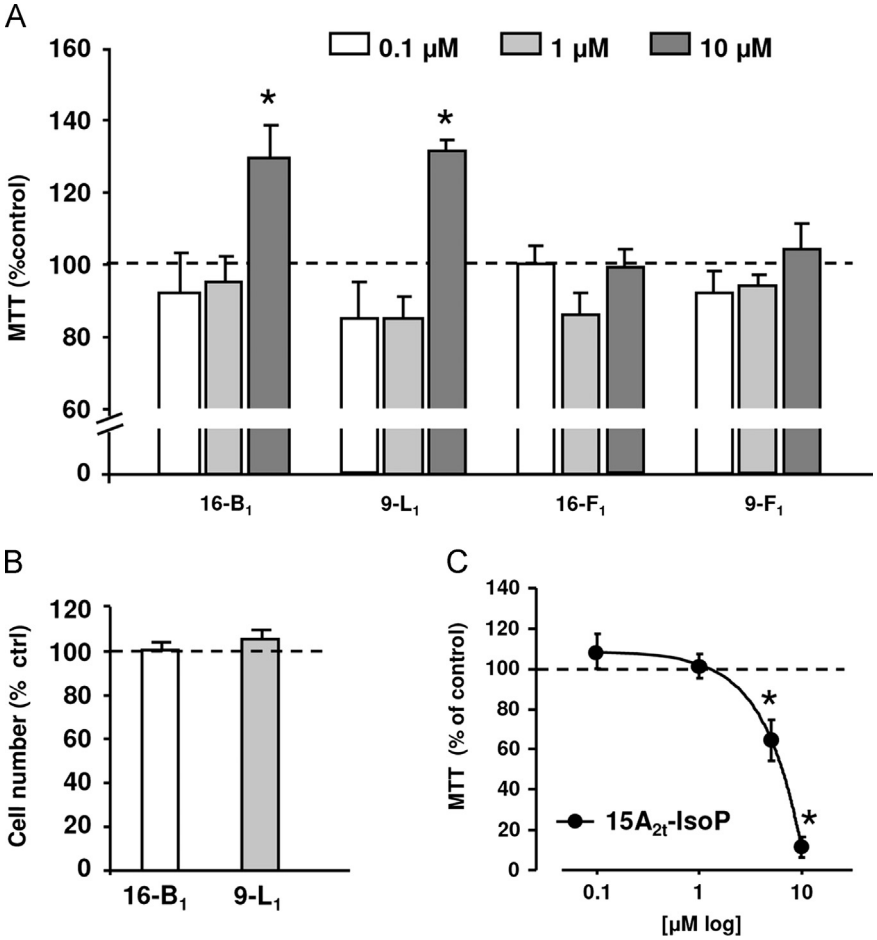

Fig. 2. Effects of B_1- and F_1-PhytoP's on SH-SY5Y cells. (A) Cell viability of SH-SY5Y cells after 24 h treatment with 0–10 μM PhytoP's, determined by MTT assay. Data are expressed as the percentage of MTT reduction in untreated control cultures. (B) Cell number in cultures after 24 h treatment with 10 μM 16-B_1 and 9-L_1, determined by CV assay and expressed as a percentage of untreated control cultures (dashed line). (C) Cell viability of SH-SY5Y cells after 24 h treatment with increasing concentrations of the cyclopentenone isoprostane 15-A_{2t}-IsoP, determined by MTT assay and expressed as a percentage of untreated control cultures (dashed line). Data are the mean ± SEM, from $n = 4$–8 independent experiments. *$p < 0.05$ versus control cultures.

interaction of PPAR-γ with the ligand. Fig. 8E shows that PPAR-γ immunoreactivity was mainly localized to the nucleus only when cells were exposed to 1 μM 16-B_1-PhytoP, and not the biologically inactive *ent*-9-L_1 PhytoP.

Discussion

In this study we have shown that B_1-PhytoP's are biologically active in experimental models of immature cells of the central nervous system. In particular, we tested the activities of the two B_1-PhytoP regioisomers (16-B_1 and 9-L_1) and found that they were equally effective in increasing the metabolic activity of SH-SY5Y cells, used for modeling an early neuronal differentiation stage. In contrast, the two F_1-PhytoP regioisomers (16-F_1 and 9-F_1) had no activity on these cells. Because the structural difference between B_1- and F_1-PhytoP's lies in their ring, this observation suggests that the cyclopentenone ring in B_1-PhytoP's, but not the cyclopentane ring in F_1-PhytoP's, is important for such activity. However, the cyclopentenone 15-A_2-IsoP, at the same concentration range, was very effective in inducing neuronal cell death, as previously described [17], thus indicating that other structural regions of B_1-PhytoP's contribute to their biological profile and that compounds generated through analogous nonenzymatic free radical-catalyzed pathways from ALA or AA are endowed with opposite biological activities.

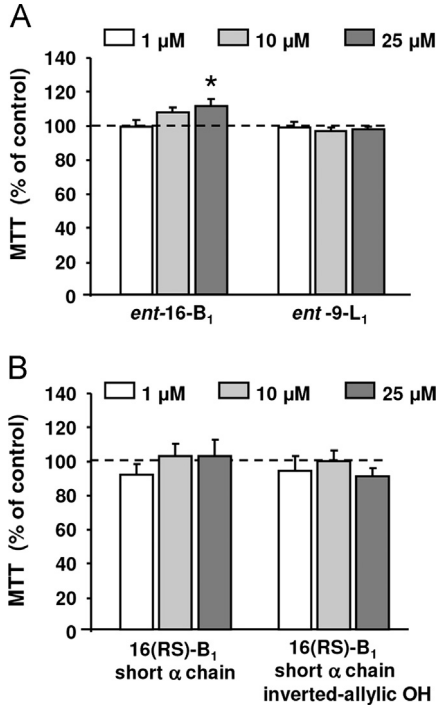

Fig. 3. Effects of B_1-PhytoP enantiomers and derivatives on SH-SY5Y cells. Cell viability of SH-SY5Y cells after 24 h treatment with 0–25 μM (A) *ent*-16-B_1 or *ent*-9-L_1 or (B) B_1-PhytoP derivatives with short α-chain with alternative allylic alcohol migration, indicated as 16(RS)-B_1 short chain and 16(RS)-B_1 short chain inverted allylic-OH, respectively. Data are expressed as a percentage of MTT reduction in untreated control cultures (ctr); $n = 4$ independent experiments; $^*p < 0.05$ versus control cultures.

The relation between biological activity and chemical structure was further investigated by testing on SH-SY5Y cells a panel of compounds comprising 16-B_1-PhytoP and 9-L_1-PhytoP enantiomers and two B_1-PhytoP derivatives characterized by a short side chain and alternative allylic alcohol migration. Of the whole panel, only the 16-B_1-PhytoP enantiomer (*ent*-16-B_1-PhytoP) retained the activity, suggesting that the allylic configuration and the position of the carboxyl group with respect to the cyclopentenone ring are two additional important factors controlling B_1-PhytoP bioactivity.

The next aim of our study was to investigate whether the B_1-PhytoP-induced increase in SH-SY5Y metabolic activity could result in cell protection against oxidant damage. For these experiments, SH-SY5Y cells were exposed to H_2O_2 at a concentration reducing their metabolic activity by about 50% to fully appreciate any protective or detrimental effect of B_1-PhytoP's. We found that 16-B_1-PhytoP, 9-L_1-PhytoP, and *ent*-16-B_1-PhytoP fully protected cells from H_2O_2 damage, whereas *ent*-9-L_1-PhytoP was devoid of activity, thus confirming the role of allylic configuration in B_1-PhytoP bioactivity. The lack of protection by *ent*-9L_1-PhytoP against H_2O_2 also ruled out the hypothesis that neuroprotection observed with the other three compounds could be due to a direct scavenging effect of tetrasubstituted cyclopentenone B_1-PhytoP's.

Interestingly, B_1-PhytoP's had no effect on differentiated SH-SY5Y cells, either on their metabolic activity or against H_2O_2-induced damage. Furthermore, B_1-PhytoP's did not protect OP cultures from H_2O_2-induced damage, but increased their metabolic activity and promoted OP differentiation to OLs, suggesting that the biological activity of these compounds is dependent on the cell type and on their state of differentiation.

To gain some insights into the molecular mechanisms triggered by B_1-PhytoP in SH-SY5Y cells we focused on the nuclear receptor PPAR-γ, a ligand-dependent transcription factor involved in the control of several important functions such as metabolism homeostasis,

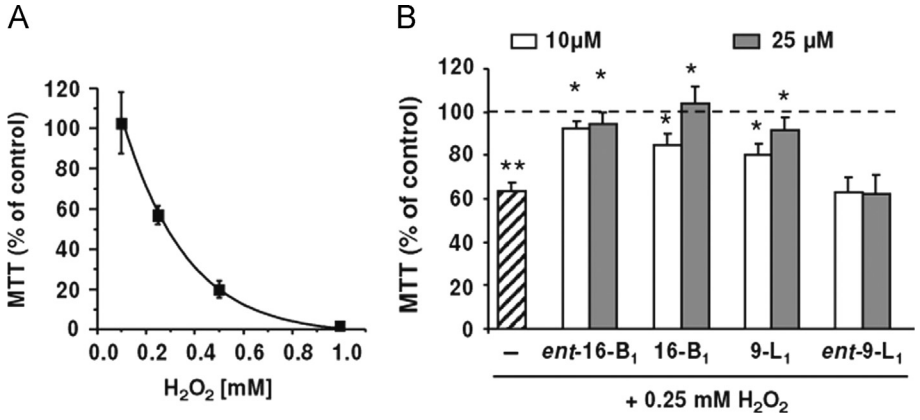

Fig. 4. Effects of B_1-PhytoP's on oxidant injury in SH-SY5Y cells. (A) Cell viability of SH-SY5Y cells after 24 h treatment with increasing H_2O_2 concentrations, determined by MTT assay. (B) Cell viability of SH-SY5Y cells exposed for 24 h to 0.25 μM H_2O_2, alone or in combination with 10 μM 16-B_1 or 9-L_1 or their enantiomer *ent*-16-B_1 or *ent*-9L_1, determined by MTT assay and compared to control cultures. Data are the mean ± SEM, from $n = 4$–6 independent experiments. $^*p < 0.05$ versus H_2O_2-treated cultures; $^{**}p < 0.05$ versus untreated control cultures (dashed line).

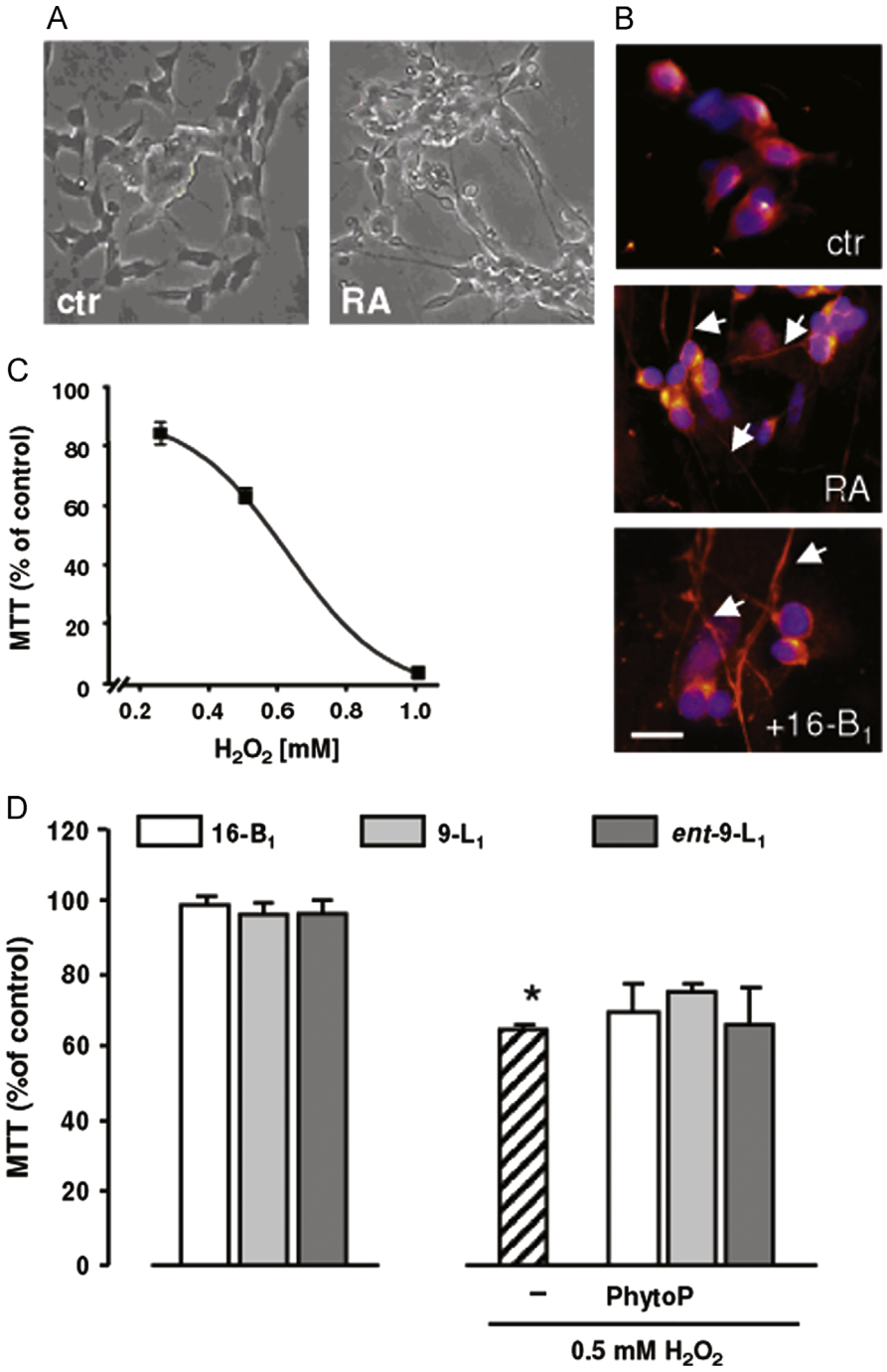

Fig. 5. Differentiated SH-SY5Y cells are resistant to B_1-PhytoP activities. (A) Bright-field images of cells cultured for 5 days in the absence (ctr) or in the presence of retinoic acid (RA), added at day 1 and day 3 as described under Materials and methods. (B) High-magnification images of nuclear stain by Hoechst and MAP2 immunostaining in control and RA-treated cells. Arrows indicate expression of MAP in neurites of differentiated cells. MAP2 expression and neurites were even more pronounced in RA-treated cells receiving 10 μM 16-B_1 for the last 24 h. Scale bar, 50 μm. (C) Cell viability of differentiated SH-SY5Y cells after 24 h treatment with increasing H_2O_2 concentrations, determined by MTT assay. (D) Cell viability of differentiated SH-SY5Y cells after 24 h treatment with 10 μM 16-B_1, 9-L_1, or *ent*-9L_1 (left) or exposed to 0.5 mM H_2O_2 alone or in combination with 10 μM 16-B_1, 9-L_1, or *ent*-9L_1, determined by MTT assay and compared to differentiated control cultures. Data are the mean ± SEM, from $n = 4$ independent experiments. $^*p < 0.05$ versus untreated control cultures.

inflammation, immunity, and cell differentiation [21]. A broad range of structurally different compounds have been shown to activate PPAR-γ, most likely because the large ligand-binding cavity allows a relatively free nonspecific interaction with the ligand. Several of the natural compounds with PPAR-γ agonistic activity are dietary lipids and their oxidized derivatives, which exhibit higher binding affinity than the unoxidized counterparts [22]. Of interest for our study, ALA has been shown to stimulate PPAR-γ at concentrations ranging from 20 to 100 μM in human renal cell carcinoma and monocytic cell lines [23,24], whereas E_1-PhytoP triggers PPAR-γ activation at a concentration as low as 1 μM in human dendritic cells [12]. In our study, the presence of the PPAR-γ antagonist GW9662 abolished the effects of 16-B_1-PhytoP, indicating the ability of the compound to trigger PPAR-γ activation at 1–10 μM concentrations in both neuronal SH-SY5Y cells

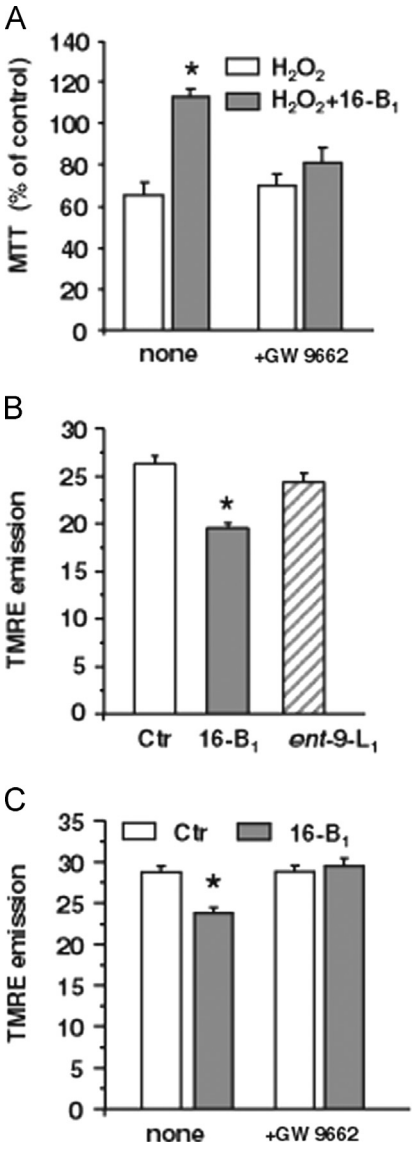

Fig. 6. PPAR-γ activation is necessary for 16-B_1-PhytoP activities on SH-SY5Y cells. (A) Cell viability of SH-SY5Y cells exposed for 24 h to 0.25 μM H_2O_2 alone or in combination with 10 μM 16-B_1, in the absence or in the presence of the PPAR-γ antagonist GW9662 (1 μM), determined by MTT assay and compared to control cultures. Data are the mean ± SEM, from $n = 3$ independent experiments; $^*p < 0.05$ versus H_2O_2-treated cultures. (B) Mitochondrial membrane potential (mMP) in cultures exposed to 10 μM 16-B_1 or *ent*-9-L_1 for 24 h; mMP was measured by using the potentiometric dye TMRE in single-cell video-imaging experiments. Data are the mean ± SEM, expressed as arbitrary units, from $n = 3$ independent experiments; $^*p < 0.05$ versus control cultures. (C) mMP in cultures exposed to 10 μM 16-B_1 alone or in combination with the PPAR-γ antagonist GW9662, measured as in (B). Data are the mean ± SEM, from $n = 3$ independent experiments; $^*p < 0.05$ versus control cultures.

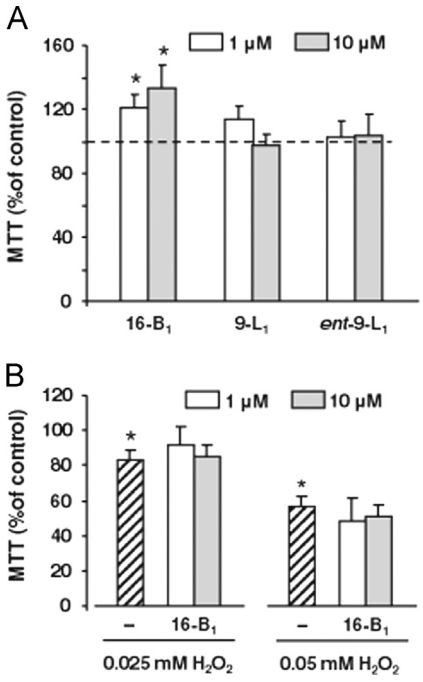

Fig. 7. Effects of B_1-PhytoP's on oligodendrocyte progenitors. (A) Cell viability of OPs after 24 h treatment with 10 μM 16-B_1, 9-L_1, or *ent*-9-L_1, determined by MTT assay. (B) Cell viability of OP cultures exposed for 24 h to 25 or 50 μM H_2O_2 alone or in combination with 1 or 10 μM 16-B_1, determined by MTT assay and compared to control cultures. Data presented in (A) and (B) are expressed as percentage of control cultures and are the mean ± SEM, from $n = 4$–6 independent experiments; $^*p < 0.05$ versus untreated control cultures.

and OP cultures. The activation of PPAR-γ is further indicated by its nuclear translocation upon stimulation with 16-B_1-PhytoP but not the biologically inactive *ent*-9-L_1-PhytoP.

Both SH-SY5Y cells and OPs constitutively express PPAR-γ. In line with our results, PPAR-γ agonists (pioglitazone and 15d-PGJ_2) prevented the death of differentiated SH-SY5Y cells exposed to transient glucose deprivation [25] and promoted OP differentiation [14,26].

In the search for a mechanism of protection against H_2O_2 in SH-SY5Y cells, we did not find any effect of B_1-PhytoP on the expression of genes involved in free radical scavenging, such as catalase or Cu/Zn- and Mn-superoxide dismutases, but we observed a moderate reduction in the mMP, an effect that was prevented by GW9662. A slight degree of depolarization within the inner membrane of mitochondria might play a protective role by attenuating the production of reactive oxygen species (ROS) by these organelles, thus rendering cells more fit to face an oxidant injury [20]. Depolarization of the inner membrane, reduced ROS production, and protection from brief anoxia–reoxygenation have been reported in isolated heart mitochondria of mice receiving a 3-week diet enriched with pioglitazone [27]. Interestingly, this study demonstrated that the effects of pioglitazone are related to the increased expression of the mitochondrial protein UCP-2, known to be under the control of PPARs [27]. It can be envisaged that a similar protection could occur in B_1-PhytoP-treated SH-SY5Y neuroblasts exposed to H_2O_2. SH-SY5Y cells are widely used to study oxidative stress-induced cell damage: the molecular mechanisms triggered by H_2O_2 or other toxic agents are complex and specific for undifferentiated or RA-differentiated cells [16,28]. In particular, the mitochondrial response seems different in neuroblasts and RA-differentiated cells; as an example, mitochondrial depolarization was faster and more pronounced in undifferentiated SH-SY5Y than in RA-differentiated cells exposed to 6-hydroxydopamine, suggesting that neuronal cells acquire mitochondria-protective mechanisms during differentiation [28]. This would explain the ability of B_1-PhytoP's to protect undifferentiated but not RA-differentiated SH-SY5Y cells. Along this line, it is interesting to note that pioglitazone did not affect mMP in differentiated SH-SY5Y cells [25]. Furthermore, 16-B_1-PhytoP added to differentiating SH-SY5Y cells (at 4 DIV) accelerated the formation of neuritis, as previously described for the PPAR-γ agonist pioglitazone [19].

In OP cultures the lack of protection against a mild to moderate H_2O_2 damage could be related to the nonsignificant effect on mMP. In agreement with these results, we have previously shown that

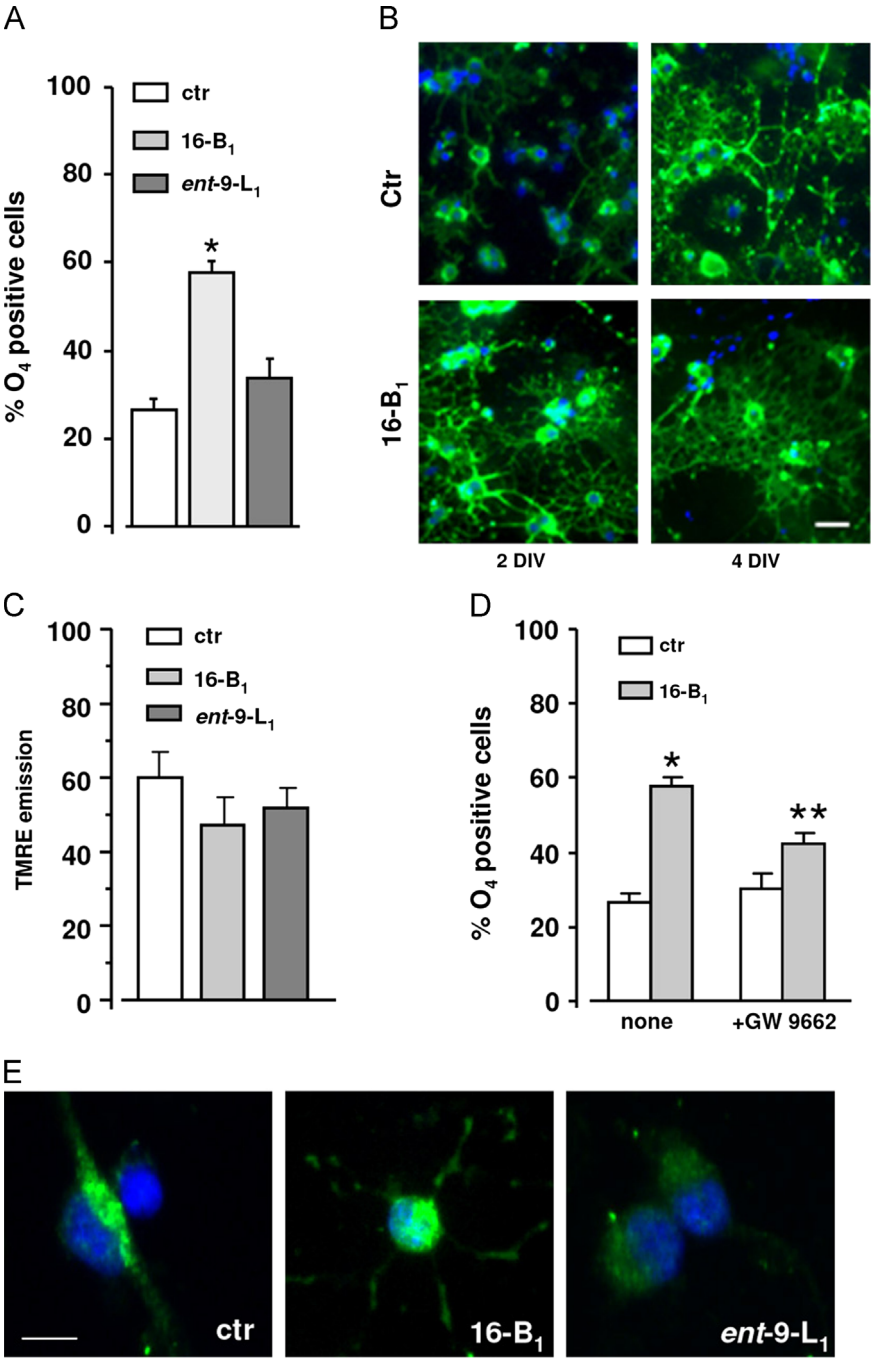

Fig. 8. 16-B_1-Phyto induces oligodendrocyte differentiation through mechanisms involving PPAR-γ activation. (A) OP cultures were treated for 24 h with 1 μM 16-B_1 or *ent*-9-L_1. OP differentiation to immature OLs was determined by evaluating the percentage of cells expressing O_4, a typical marker for this OL developmental stage. Nuclei were stained using Hoechst 33258. Data are given as the percentage of positive cells over the total cell number under each condition and are the mean ± SEM from $n = 4$ independent experiments; $^{*}p < 0.05$ versus untreated control cultures and versus *ent*-9-L_1-treated cultures. (B) Images of Hoechst 33258 nuclear staining (blue) and O_4 immunostaining (green) in cultures grown for 24 h (2 DIV) or 72 h (4 DIV) under control (Ctr; top) and 16-B_1-treated (bottom) conditions. Images show the more complex morphology of 16-B_1-treated cells, indicating a more mature developmental stage. Scale bar, 50 μm. (C) Mitochondrial membrane potential in cultures exposed to 10 μM 16-B_1 or *ent*-9-L_1 for 24 h; mMP was measured by using the potentiometric dye TMRE in single-cell video-imaging experiments. Data are the mean ± SEM and are expressed as arbitrary units; $n = 4$ independent experiments. (D) Percentage of O_4-positive cells in cultures treated for 24 h with 1 μM 16-B_1 alone or in the presence the PPAR-γ antagonist GW9662 (1 μM). Data are the mean ± SEM from $n = 4$ independent experiments; $^{*}p < 0.05$ versus untreated control cultures; $^{**}p < 0.05$ versus 16-B_1-treated cultures. (E) Images of Hoechst 33258 nuclear staining (blue) and PPAR-γ immunostaining (green) in cultures grown for 24 h in the absence (ctr) or in the presence of 1 μM 16-B_1 or *ent*-9-L_1. Images show PPAR-γ nuclear localization only in cultures exposed to 16-B_1. Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PPAR-γ agonists do not influence mMP in OL cultures, although they accelerate OP differentiation to OLs through a mechanism involving mitochondrial functions, such as the mitochondrial respiratory chain activity and oscillatory Ca^{2+} wave regulation [26].

Altogether, these observations suggest that B_1-PhytoP bioactivities are cell-type-dependent and restricted to immature cells. Indeed, we have shown that B_1-PhytoP's, through mechanisms involving PPAR-γ, can affect neuroblasts and OPs thereby conferring neuroprotection against oxidant injury and promoting myelination, respectively. Being dietary components, PhytoP's should be included in the large group of nutraceuticals that can activate PPAR-γ and that could be exploited to promote health benefits

[22]. In the past decade, there has been an increasing interest in the anti-inflammatory and neuroprotective activities of PPAR-γ agonists and their therapeutic potential in a variety of brain disorders [21,29,30]. Nonetheless, the toxicity associated with some synthetic agonists, including the antidiabetic glitazones, has fostered the search for alternative strategies for targeting PPAR-γ. In particular, there has been an increasing interest in natural compounds that can be integrated into the daily diet and provide a complementary treatment for chronic diseases including neurological diseases such as multiple sclerosis [31].

Finally, the high content of PhytoP's in pollen suggests that these molecules can gain access to the brain through the nasal mucosa, via the olfactory epithelium, which represents a direct route from the nose to the brain along the olfactory and trigeminal nerve pathways, or via the vascular route into the blood and across the blood–brain barrier [32]. Through these routes B_1-PhytoP's could reach brain areas in which stem and progenitor cells reside and influence their fate.

Our study highlights the importance of a detailed knowledge of the biological activities of PhytoP's in mammalian systems to appreciate the potential benefit and detriment of accidental (pollen) or deliberate (diet and parenteral lipid nutrition) exposure to these oxidized lipids.

Acknowledgments

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