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Trolox protection of myelin membrane in hydrogen peroxide-treated mature oligodendrocytes

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Abstract
Oligodendrocytes have the highest rate of metabolic activity in the brain and are highly vulnerable to oxidative stress. In this work we determined the protective effect of Trolox, a water-soluble analogue of vitamin E, and insulin, a peptide shown to be neuroprotective, in oligodendrocyte lesion induced by hydrogen peroxide (H_2O_2). Exposure of primary cultures of rat oligodendrocytes to H_2O_2 dose-dependently decreased their reducing capacity, as determined by the MTT assay. H_2O_2 (100 μM) had no effect on Bax levels, active-caspase-3, DNA fragmentation or lactate dehydrogenase (LDH) leakage. Nevertheless, under these conditions, H_2O_2 decreased the levels of myelin basic protein (MBP), used as a marker for oligodendrocyte myelin membrane. Treatment with insulin alone increased MBP levels, but no changes were observed in the presence of insulin plus H_2O_2. In contrast, incubation with Trolox completely prevented H_2O_2-induced decrease in MBP expression, suggesting that vitamin E analogues may prevent against oligodendrocyte oxidative damage.

Keywords: *Cell death, hydrogen peroxide, myelin basic protein, oligodendrocytes, oxidative stress, Trolox*

Introduction

In the central nervous system (CNS), oligodendrocytes produce myelin, a lipid-rich biological membrane that forms multilamellar, spirally-wrapped sheets around axons. Myelination of axons greatly increases the conduction velocity of the nervous impulse, and the devastating neurological effects caused by demyelinating CNS diseases such as multiple sclerosis (MS) illustrate the importance of the process.

Oligodendrocytes have the highest rate of oxidative metabolic activity of any cell in the brain, implicating elevated oxygen consumption. Oligodendrocytes also have a particular high intracellular iron concentration [1,2], a high content in polyunsaturated fatty acids and low levels of reduced - glutathione [2,3], contributing for their vulnerability to oxidative stress. Thus, it is thought that oxidative damage to oligodendrocytes can retard or inhibit myelination [2,4].

Oxygen and nitrogen free radicals have been implicated in the pathogenesis of some neurodegenerative diseases, including MS. This disease is characterized by perivenous infiltration of lymphocytes and macrophages, which, together with activated microglia, have been implicated as mediators of demyelination, axonal injury and neuronal loss [5–10]. Indeed, one of the most abundant sources of reactive oxygen species (ROS) is the respiratory

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burst system of activated microglia, which releases large quantities of ROS, especially superoxide anions ($O_2^{\cdot-}$) [10], leading to oxidative stress that may affect both the oligodendrocytes and the neurons. Numerous studies of patients with MS and direct examination of MS plaques have shown increased free radical activity, and/or deficiencies in important antioxidant enzymes (superoxide dismutase and glutathione peroxidase), along with decreased levels of non-enzymatic antioxidants, such as glutathione, vitamin E and uric acid, compared with healthy controls [10,11]. Therefore, administration of antioxidants might prevent free radical-mediated tissue destruction in MS [10,12,13]. Insulin has been reported to be involved in the regulation of brain metabolism, neuronal growth and differentiation, and to have a neuroprotective role [14], by preventing oxidative stress-mediated decrease in energy metabolism and neuronal death in cultured cortical neurons [15,16]. In addition, insulin also interacts with insulin-like growth factor-1 receptors, previously shown to be present in oligodendrocytes from rat brain [17].

In the present study, we analyzed the effect of H_2O_2 on cell viability and myelin membrane homeostasis by determining apoptotic and necrotic features of cell death and the levels of myelin basic protein (MBP) in primary cultures of rat oligodendrocytes. MBP is one of the major proteins of CNS myelin and constitutes as much as 30% of the total protein [18]. In order to test if the damage caused by increased generation of ROS could be prevented, we tested the effect of the antioxidant Trolox, a water-soluble analogue of vitamin E and insulin against oxidative injury induced by H_2O_2 in primary cultures of mature oligodendrocytes.

Materials and methods

Materials

MEM-Hepes, DMEM and DMEM-F12 1:1 ratio media, papain, L-cysteine, DNAse I Type IV, L-glutamine (L-Gln), penicillin/streptomycin (P/S), glycine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Trolox and insulin were from Sigma Chemical Co. (St. Louis, MO, USA). B-27 supplement was purchased from GIBCO (Paisley, UK). Fetal calf serum (FCS) was from BioWhittaker Molecular Applications (Rockland, MD USA). Anti-MBP was from Serotec (Oxford, UK); anti-Bax was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); anti-active caspase 3 was from Promega (Madison, WI, USA); anti-NG2 and anti-GAPDH (3-phosphate-glyceraldehyde dehydrogenase) were from Chemicon (Temecula, CA, USA). Hoechst 33342, Mito-Tracker Red and Mito-Tracker Green were from Molecular Probes (Eugene, OR, USA). Dako fluorescent mounting medium was from DakoCytomation (Glostrup, Denmark). All other reagents were of analytical grade.

Primary culture of mature oligodendrocytes from rat brain

Primary cultures of oligodendroglial cells were prepared from the brains of neonate Wistar rats by following the method of McCarthy and Vellis (1980) [19], with minor modifications. Brain cortices were dissected free of meninges, minced two times with a Gilson pipette and incubated for 40 min at 37°C in an enzymatic solution containing 30 U/ml papain, 0.24 mg/ml L-cysteine and 40 μg/ml DNAase I type IV in MEM-Hepes. Then, DMEM supplemented with 10% FCS, 200 mM L-Gln and P/S (1:100) were added to the cell suspension in order to stop the digestion. The solution was centrifuged (Sigma 3K10 Laborzentrifugen) at 1200 rpm during 5 min and the supernatant was removed. Fresh DMEM media containing 10% FCS, L-Gln and P/S was added and the tissue was ground with 1 ml pipette until no aggregates were visible. Cells were cultured at a density of two brains per 75 cm^2 flask, precoated with poly-L-lysine (0.1 mg/ml), in DMEM supplemented with 10% FCS, L-Gln and P/S and maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. Media was renewed every 4 days. After 10 days in culture, the cells had separated into two layers: a basal monolayer consisting primarily of astrocytes and some fibroblasts and a top layer containing oligodendrocyte precursor cells (OPCs) and microglia. The loosely attached microglia were removed during a preliminary 1 h shake on an orbital shaker (GFL 3032/3033 shaking incubator) at 170 rpm/min, at 37°C, after which the supernatant and loose cells were discarded and 10 ml of fresh media were added. The flasks were then shaken for further 24 h at 170 rpm/min. During this period the majority of top layer cells, mostly OPCs and remaining microglia, were detached from the monolayer. Microglial contaminants were then removed by plating the media resulting from the previous shaking in non-coated plastic petri dishes (Corning, USA) for 20 min at 37°C, during which the adhesive microglia stuck down, whereas the OPCs stayed unattached. The remaining cell suspension was then centrifuged at 1200 rpm for 5 min and resuspended in DMEM-F12 1:1 ratio media supplemented with B-27 1:50, 0.5% FCS and P/S, and plated in poly-L-lysine coated plates. The cells differentiated *in vitro* for 5 days, in an atmosphere of 5% CO_2 (37° C), as examined by immunocytofluorescence using anti-NG2 (an OPC marker) and anti-MBP (a marker of mature oligodendrocytes) (Figure 1(A) (a),(b)).

By following the method of McCarthy and Vellis (1980) [19], after removing the culture media containing cells in suspension from the 75 cm^2 flask, a few proliferative OPCs remained attached to the basal monolayer. Thus, 10 ml of fresh media DMEM with 10% FCS, L-Gln and P/S were added to the flasks and the cells were cultured at 37°C in an

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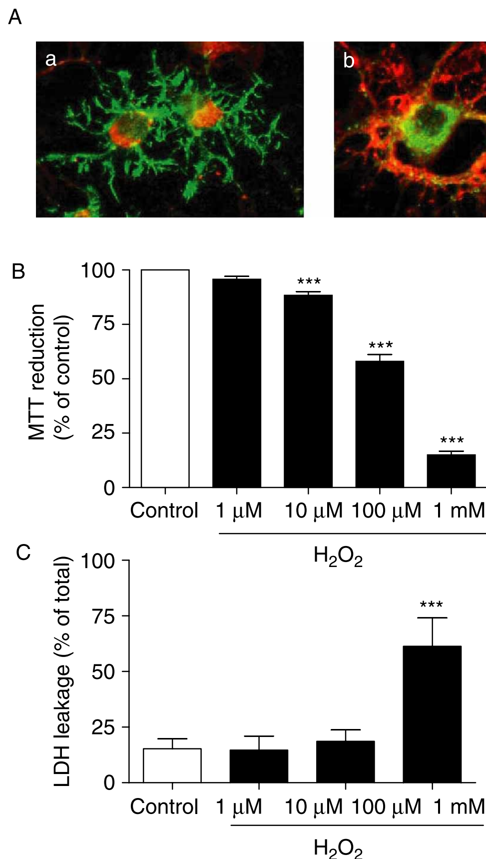

Figure 1. Cell viability of differentiated oligodendrocytes in culture subjected to H_2O_2 treatment. (A) The differentiation status of oligodendrocyte cultures was characterized by immunocytofluorescence. (a) Progenitor cells were labelled with anti-NG2 (green) and Mito-Tracker Red at 2 days in culture; (b) differentiated oligodendrocytes at 4 days in culture were stained with anti-MBP (red) and Mito-Tracker Green. Note the preferential localization of mitochondria in the perinuclear region in both OPCs (a) and mature oligodendrocytes (b). Cell viability was determined by following the changes in cell reducing capacity by the MTT assay (B) or by determining the release of intracellular LDH (C), an index of membrane integrity. Oligodendrocytes were incubated with increasing concentrations of H_2O_2 (1–1000 μM) for 24 h. Data are the mean ± SEM of nine independent experiments, performed in duplicates. Statistical significance: $***P < 0.001$, compared to the control.

atmosphere of 5% CO_2 for further 7 days (media was renewed at day 4). After a week, the top layer was again populated by OPCs and some microglial cells. The same procedure to remove contaminant microglia was followed (as described above), and the OPCs were cultured in DMEM-F12 1:1 media, supplemented with B-27 1:50, 0.5% FCS and P/S.

Cells were cultured at 6×10^5, 4×10^5 and 1×10^5 cells/cm^2 for Western blot, lactate dehydrogenase (LDH) leakage and MTT assay experiments, respectively. For immunocytochemistry assays, oligodendrocytes were plated in 16 mm poly-L-lysine coated glass coverslips at a density of 0.5×10^5 cells/coverslip.

Incubation of oligodendrocytes

Oxidative injury was induced in the presence of H_2O_2 (1–1000 μM), incubated for 24 h in culture media, at 37°C, in an atmosphere of 5% CO_2. When applied, Trolox was used at a final concentration of 10 μM from a 10 mM stock solution prepared in DMSO. Insulin was prepared from a 0.84 mM stock solution in water and used at a final concentration of 1 μM. Trolox and insulin were added to the culture media 30 min prior to incubation with H_2O_2.

MTT assay

The reduction capacity of oligodendrocytes was evaluated by a colorimetric assay for cell survival, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), according to the method described by Mosmann (1983) [20]. MTT (0.5 mg/ml) was prepared in sodium saline solution. The incubation media was discarded and the cells washed twice with sodium solution. Then, MTT was added to the cell culture and incubated for 2 h, at 37°C, in the dark. When taken up by living cells, MTT is converted to water-insoluble blue product (formazan). The precipitated dye was dissolved in 40 mM HCl in isopropanol and colorimetrically quantified at 570 nm in an Elisa microplate reader (SLT Spectra).

Lactate dehydrogenase (LDH) measurements

Membrane integrity was assessed by determining the release of the cytoplasmic enzyme LDH from the injured cells into the extracellular medium. This procedure represents a common method to determine membrane leakage and cellular damage, characteristic of necrotic cell death. Briefly, the extracellular medium was recovered and kept on ice (0–4°C). Intracellular LDH was obtained by two cycles of freezing at −80°C after adding a hypotonic solution containing 10 mM HEPES and 0.01% Triton X-100. Both intracellular and extracellular samples were centrifuged at 14,000 rpm (Eppendorf Centrifuge 5417R), for 10 min, at 4°C. The supernatant was recovered and used to measure LDH activity, which was determined spectrophotometrically in the presence of 9.76 mM pyruvate and 0.244 mM NADH. The rate of conversion of reduced NADH to NAD^+ was followed at 340 nm, in a Perkin Elmer lambda 2 UV/VIS spectrophotometer. LDH leakage was expressed as a percentage of total activity (% of LDH release = extracellular LDH/extracellular LDH + intracellular LDH) [21].

Nuclear morphology assay

The nuclear morphology of differentiated oligodendrocytes was analyzed by fluorescence microscopy

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of living cells using Hoechst 33342. After incubation for 24 h with 100 μM H_2O_2 in the absence or in the presence of 10 μM Trolox, the culture medium was removed and the cells were washed briefly in PBS, fixed in methanol–acetone (1:1) for 10 min and incubated with 4 μg/ml Hoechst for 10 min. Then, the coverslips were fixed to glass slide with DAKO mounting medium. Fluorescence of stained chromatin was visualized using a fluorescence microscope (Axioskop 2 Plus Zeiss) and the images were acquired with the Axiovision software 4.2.

Immunocytofluorescence

The culture medium was removed and the cells were washed three times in phosphate buffer solution (PBS, in mM: 137 NaCl, 2.7 KCl, 1.4 K_2HPO_4, 4.3 Na_2HPO_4, pH 7.4). To stain the mitochondria, some cells were incubated with Mito-Tracker Green or Mito-Tracker Red (0.75 μM) in sodium saline solution (in mM: 132 NaCl, 4 KCl, 1.2 NaH_2PO_4, 1.4 $MgCl_2$, 10 Hepes, 6 glucose and 1 $CaCl_2$, pH 7.4). Then, the cells were fixed with methanol–acetone (1:1) for 10 min. The oligodendrocytes were further incubated with 20 mM glycine (in PBS) for 10 min. Cell membrane was permeabilized with 0.1% saponin (in PBS) (PBS 0.1% S) during 30 min. After a wash with PBS 1% S, rat anti-MBP (1:75) and rabbit anti-NG2 (1:200) were prepared in PBS 1% S and incubated for 1 h at room temperature. Then, three washes were carried out with PBS 0.1% S and the secondary antibodies anti-rat cy3 (1:300) and anti-rabbit IgG Alexa-fluor 488 (1:200) were incubated in PBS 0.1% S, for 1 h, at room temperature. The coverslips were mounted using DAKO solution. Cells were visualized using a confocal microscope Biorad MRC600. Final images were treated with confocal assistant application (CAS).

For analysis of caspase-3, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (in PBS) for 5 min, washed three times in PBS, and incubated with a blocking buffer containing 3% BSA in PBS/0.1% Tween for 2 h. Then, the cells were incubated with anti-active caspase-3 (1:250) and anti-MBP (1:75) during 2 h at room temperature. Caspase-3 and MBP were visualized by using the secondary antibody anti-rabbit IgG Alexa Fluor 488 (1:200) and anti-rat cy3 (1:300), respectively. Oligodendrocytes were visualized using a fluorescence microscope (Axioskop 2 Plus Zeiss) and the images were acquired with the Axiovision software 4.2.

Western blotting analysis

The culture medium was removed, and the cells were lysed with a buffer containing (in mM): 100 NaCl; 20 Tris; 2 EDTA; 2 EGTA, supplemented with 0.1 mM PMSF and 1:1000 of protease inhibitor cocktail (chymostatin, leupeptin, antipapain and pepstatin, 1 mg/ml) plus 0.5% Triton X-100, on ice. The protein was measured by using the BioRad protein assay and the lysates were sonicated briefly and heated at 100°C for 5 min. Then, the samples were submitted to electrophoresis on a 12% SDS-PAGE gel at 90 V and electroblotted onto polyvinylidene difluoride (PVDF) membranes in CAPS/methanol 10%, during 90 min at 0.75 A. The membranes were blocked with 5% skim milk in TBS (25 mM Tris–HCl, pH 7.6, 150 mM NaCl)/0.1% Tween, during 60 min and incubated overnight at 4°C with rat anti-MBP (1:500) or rabbit anti-Bax (1:250). Mouse anti-GAPDH (1:5000) was used to normalize the amount of protein per lane. Then, the membranes were washed three times with TBS-T 1% containing skim milk, and further incubated with secondary antibodies, anti-rat IgG (1:5000) or anti-rabbit IgG (1:20,000), during 2 h. Proteins were visualized by using an enhanced chemifluorescence reagent (Amersham Biosciences, Buckinghamshire, UK) and the bands were detected with the BioRad VersaDoc 3000 Imaging System.

Data analysis

The results are the mean ± SEM of the number of experiments indicated in the figure legends. Data were analyzed using GraphPad Prism version 4.00 for Windows (Graph Pad Software, San Diego, CA, USA). Statistical analysis was performed by using the Student's *t*-test, or one-way ANOVA with Tukey's multiple comparison post-test ($P < 0.05$ was considered significant).

Results

Characterization of cultured oligodendrocytes and changes in cell viability upon exposure to H_2O_2

The different stages of oligodendrocyte differentiation can be defined using antibodies recognizing some of the major developmental markers for the oligodendrocyte lineage. NG2 proteoglycan, an integral membrane chondroitin sulfate proteoglycan, is expressed by oligodendrocyte progenitors [18,22], whereas MBP is expressed by mature myelinating cells [18]. After 2 days in culture, OPCs expressed the marker NG2 and displayed characteristically branched morphology (Figure 1(A) (a)). After 4 days in culture, differentiated oligodendrocytes expressed MBP and formed myelin membranes (Figure 1(A) (b)). We also analyzed the distribution of mitochondria during oligodendrocyte differentiation using Mito-Tracker Red and Mito-Tracker Green. From day 0 (Pereira and Rego, unpublished data) to day 2 (Figure 1(A) (a)), the mitochondria appeared highly concentrated in a perinuclear region. With increasing oligodendrocyte differentiation, mitochondria distributed not only

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around the nucleus, but also through the cellular branches (Figure 1(A) (b)).

In order to evaluate the effect of H_2O_2 on the viability of mature oligodendrocytes, we followed the changes in cellular reduction capacity by the MTT assay, and the alterations of plasma membrane integrity by LDH leakage. Contrary to prior publications, in which short H_2O_2 incubation periods were applied [2,23], we tested an incubation period of 24 h to mimic the extended inflammatory process occurring in MS. In MS, macrophages and activated microglia produce large amounts of ROS that are thought to result in myelin damage and oligodendrocyte and neuronal cell death [10]. Incubation of oligodendrocytes with 1 μM H_2O_2 did not significantly affect MTT reduction. However, exposure to 10–1000 μM dose-dependently decreased the capacity of oligodendrocytes to reduce MTT (Figure 1(B)). LDH release was significantly altered after exposure to high H_2O_2 concentrations (1 mM), indicating a loss of plasma membrane integrity. However, cells incubated with 10–100 μM H_2O_2 maintained similar levels of LDH leakage compared to the control (Figure 1(C)), evidencing no signs of membrane disruption commonly associated with necrotic cell death. Thus, although MTT reduction was altered by low concentrations of H_2O_2 (10 μM), only high doses of this oxidant (1 mM H_2O_2) induced cell death by necrosis.

Analysis of apoptotic features in oligodendrocytes submitted to H_2O_2

H_2O_2 was previously shown to induce oligodendrocyte cell death by apoptosis [23,24]. Therefore, we analyzed three apoptotic features, namely Bax levels, caspase-3 activation and DNA fragmentation, in the presence of 100 μM H_2O_2, a concentration shown to induce changes in oligodendrocyte reduction capacity (Figure 1(B)), although not causing cell death by necrosis (Figure 1(C)).

Bax, a member of the pro-apoptotic Bcl-2 family of proteins, integrates into the outer mitochondrial membrane where it oligomerizes and contributes to mitochondrial permeabilization [23–26]. Our results show that Bax levels do not significantly change in oligodendrocytes exposed to 100 μM H_2O_2, in the absence or in the presence of Trolox (10 μM) or insulin (1 μM) (Figure 2(A)).

Executioner caspase activation occurs at a final stage of the apoptotic process [27]. Therefore, we analyzed caspase-3 activation by immunocytochemistry, using an antibody against active-caspase 3, in oligodendrocyte cultures exposed to 100 μM H_2O_2, in the absence or in the presence of 10 μM Trolox. No evidence of active caspase-3 was found in the nucleus of H_2O_2-treated (Figure 2(B) (b)) vs. control (Figure 2(B) (a)) oligodendrocytes.

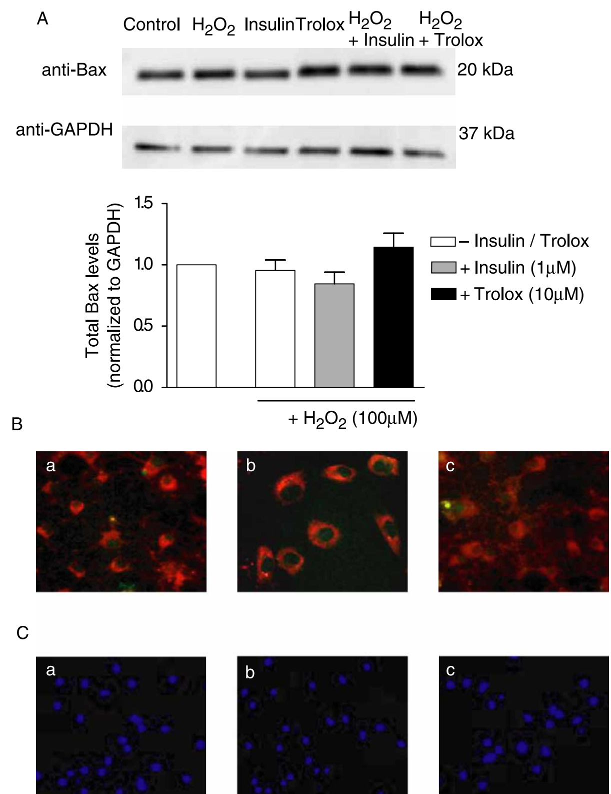

Figure 2. Analysis of apoptotic features in differentiated oligodendrocytes exposed to H_2O_2. Cultured oligodendrocytes were exposed to H_2O_2 (100 μM) in the absence or in the presence of Trolox (10 μM) or insulin (1 μM) for 24 h. (A) Oligodendrocyte extracts were analyzed by Western blotting using anti-Bax and anti-GAPDH. Data depicted in the graph are the mean ± SEM of three independent experiments. (B) Oligodendrocytes were immunostained for MBP (red) and active-caspase 3 (green) and visualized by fluorescence microscopy. (C) Oligodendrocyte nuclei were stained with Hoechst 33342 and visualized by fluorescence microscopy. In (B) and (C): (a) control, (b) 100 μM H_2O_2, and (c) 100 μM H_2O_2 plus 10 μM Trolox.

As a means to further discriminate the apoptotic cells, we analyzed the nuclear morphology of oligodendrocytes exposed to 100 μM H_2O_2. Nevertheless, data shown in Figure 2(C) did not reveal any feature of DNA fragmentation/condensation.

These data suggest that incubation of oligodendrocyte cultures with H_2O_2 (100 μM) over a period of 24 h is not sufficient to induce death by apoptosis or necrosis.

Changes in MBP expression upon exposure to H_2O_2: Effect of Trolox and insulin

Rat oligodendrocytes express four major MBP isoforms with molecular weights of 14, 17, 18.5 and 21.5 kDa, respectively [18].

By Western blot analysis we identified three MBP bands in extracts from cultured rat oligodendrocytes, corresponding to isoforms with 17, 18.5 and 21.5 kDa. The results showed a decrease in total

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MBP expression after incubation with 100 µM H_2O_2, whereas no significant differences were observed after exposure to 10 µM H_2O_2 (data not shown), a concentration that only slightly affected cell reducing capacity (Figure 1(B)). No significant changes in MBP expression were attributed to specific isoforms. Interestingly, a significant increase in MBP expression was observed in the presence of Trolox plus H_2O_2 when compared to H_2O_2 alone (Figure 3(A)). Trolox was prepared in DMSO (0.1% final concentration), which *per se* did not affect cell viability or MBP levels in control oligodendrocytes (not shown). Note that Trolox alone non-statistically increased MBP levels (Figure 3(A)). Recover of MBP levels upon exposure to Trolox suggests a protection from changes in myelin homeostasis.

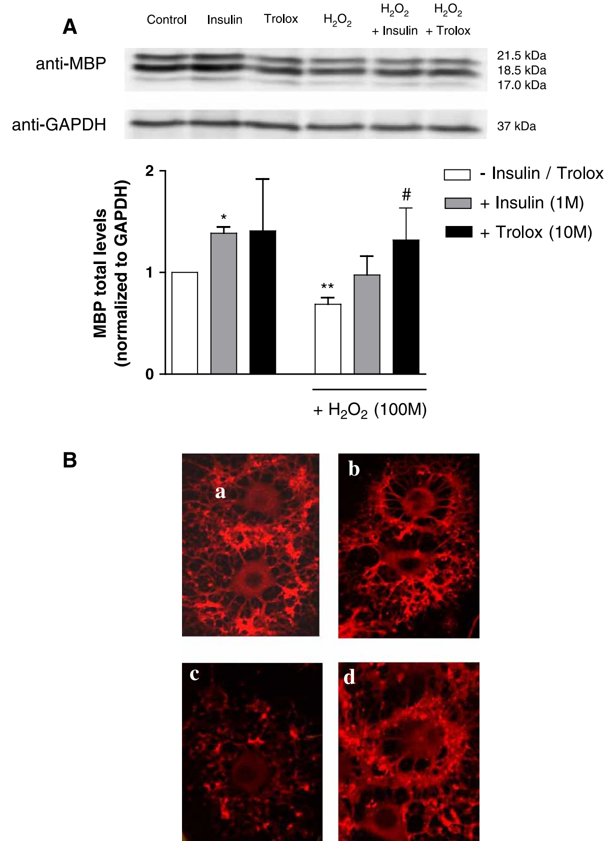

Figure 3. Trolox prevents the reduction of MBP levels in differentiated oligodendrocytes exposed to H_2O_2. Oligodendrocytes were incubated with H_2O_2 (100 µM) in the absence or in the presence of Trolox (10 µM) and insulin (1 µM) for 24 h. (A) Cell extracts were analyzed by Western blotting with anti-MBP and anti-GAPDH. Note the three bands corresponding to MBP isoforms with 21.5, 18.5 and 17 kDa. Data in the graph show the sum of the three isoforms of MBP (normalized to GAPDH), expressed as the mean ± SEM of three independent experiments. Statistical analysis: $^{*}P < 0.05$ or $^{**}P < 0.01$ compared to the control; $^{\#}P < 0.05$ compared to 100 µM H_2O_2, in the absence of insulin or Trolox. (B) Oligodendrocytes immunostained with anti-MBP were visualized by confocal microscopy. (a) Control, (b) 10 µM H_2O_2, (c) 100 µM H_2O_2, and (d) 100 µM H_2O_2 plus 10 µM Trolox.

The data also showed an increase in MBP protein levels in cells cultured in the presence of insulin, when compared to control. However, no changes in MBP protein levels were observed in oligodendrocytes cultured in the presence of insulin treated with H_2O_2, compared to oligodendrocytes treated with H_2O_2 but cultured in the absence of insulin (Figure 3(A)), suggesting that insulin does not protect oligodendrocytes from H_2O_2 lesion.

Confocal microscopy analysis of oligodendrocytes in culture upon exposure to H_2O_2 revealed that 10 µM (Figure 3(B) (b)) and particularly 100 µM H_2O_2 (Figure 3(B) (c)) induced a decrease in MBP immunocytofluorescence, compared to control cells (Figure 3(B) (a)). When added to H_2O_2-treated cells, Trolox could rescue the harmful effect of H_2O_2 and restored MBP immunofluorescence levels (Figure 3(B) (d)), therefore, confirming the results obtained by Western blotting.

Discussion

In the present study, we evaluated the toxic effects induced by H_2O_2 in primary cultures of differentiated (mature) oligodendrocytes. No evidence of cell death by necrosis or apoptosis was found upon exposure to H_2O_2 (100 µM). However, under these conditions, H_2O_2 severely affected oligodendrocyte myelin membrane homeostasis and resulted in decreased MBP protein levels. We also analyzed the effect of Trolox, a vitamin E analogue, and insulin against H_2O_2-mediated injury and, although no significant changes were observed in cultures treated with insulin, Trolox significantly prevented H_2O_2-induced decrease in MBP. These data suggest that antioxidants may be used to prevent oxidative-mediated changes in oligodendrocyte myelin membrane.

ROS can damage critical cellular components, such as DNA, proteins and membrane phospholipids, eventually leading to cell death [28–31]. Oligodendrocytes are very sensitive to oxidative stress *in vitro* due to a low capacity for antioxidant defense and the presence of high iron content [31,32]. The present study demonstrates that the H_2O_2 affects oligodendrocyte reduction capacity without causing disruption of the plasma membrane.

Previous studies demonstrated that mature cultured rat brain oligodendrocytes are specifically susceptible to free radical damage and that H_2O_2 induces cell death by apoptosis [2]. Mronga et al. (2004) [23] sustained that Bax protein is prominently expressed in mature oligodendrocytes and showed that oxidative stress causes an increase in Bax levels. Thus, the presence of Bax might be related to an enhanced susceptibility of oligodendrocytes to stress situations. Nevertheless, our data showed that under experimental conditions not involving necrotic cell death, total Bax levels were not changed. Prominent features

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of apoptosis include caspase activation and DNA condensation and fragmentation. Caspase 3 is a potent effector of apoptosis and promotes oligodendrocyte death in cultures exposed to hypoxic injury [31,33]. However, our study demonstrated that H_2O_2 (100 μM) did not cause the activation of caspase 3. In line with these results, Fragoso et al. (2004) [31] determined that H_2O_2 (0.01–0.5 mM) was not sufficient to cause an increase in caspase 3 in mature oligodendrocytes. Concordantly, features of DNA fragmentation were not observed in the present study. In addition, Fragoso et al. (2004) [31] and Baud et al. (2004) [34] found that progenitor cultured cells were more sensitive than mature oligodendrocytes to H_2O_2-induced cell death. Moreover, and according to these authors, differentiated oligodendrocytes contain more glutathione levels than progenitor cells, suggesting that a higher antioxidant content may be the basis for their resistance to cell death after exposure to H_2O_2, although, as demonstrated by our results, it might not be sufficient to completely prevent myelin membrane lesion.

Considering that MBP protein expression may serve as an index for myelin membrane homeostasis, analysis of MBP protein levels gave relevant information about oligodendrocyte susceptibility to oxidative injury. Accordingly, H_2O_2-induced decrease in cellular function was associated with a reduction in MBP expression. This reduction was prevented by the water soluble mimetic of vitamin E, Trolox. Trolox-mediated cytoprotection of oligodendrocytes was previously demonstrated [35]. Treatment with Trolox and inhibition of poly(ADP-ribose)polymerase-1 protected oligodendrocytes against damage induced by complement, which together with glutamate excitotoxicity, have been implicated in tissue damage in MS [36].

Hence, our data strongly suggest that under conditions of mild oligodendrocyte oxidative stress, the myelin sheath can be preserved in the presence of antioxidants such as Trolox. Accumulating evidence suggests that oxidative stress contributes to various diseases that affect oligodendrocytes, including MS, due to increased formation of ROS and reduced levels of gluthatione, uric acid and vitamin E, as well as of antioxidant enzymes, superoxide dismutase and gluthatione peroxidase. Importantly, vitamin E levels have been reported to be decreased in demyelinating plaques of MS brains [12,13]. Thus, substances with antioxidant capabilities, such as vitamin E or its analogues, might protect against the development of the disease.

In conclusion, the present work suggests that H_2O_2, even at relative low levels, severely affects myelin membrane homeostasis in oligodendrocytes, as evidenced by a decrease in MBP immunofluorescence and total MBP protein levels. The results also suggest that the harmful effects of the oxidative lesion may be prevented by antioxidants, emphasizing their importance in the prevention of cell injury associated with demyelinating diseases.

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