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PrP^C displays an essential protective role from oxidative stress in an astrocyte cell line derived from PrP^C knockout mice

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ABSTRACT

The PrP^C protein, which is especially present in the cellular membrane of nervous system cells, has been extensively studied for its controversial antioxidant activity. In this study, we elucidated the free radical scavenger activity of purified murine PrP^C in solution and its participation as a cell protector in astrocytes that were subjected to treatment with an oxidant. *In vitro* and using an EPR spin-trapping technique, we observed that PrP^C decreased the oxidation of the DMPO trap in a Fenton reaction system (Cu^{2+}/ascorbate/H_2O_2), which was demonstrated by approximately 70% less DMPO/OH·. In cultured PrP^C-knockout astrocytes from mice, the absence of PrP^C caused an increase in intracellular ROS (reactive oxygen species) generation during the first 3 h of H_2O_2 treatment. This rapid increase in ROS disrupted the cell cycle in the PrP^C-knockout astrocytes, which increased the population of cells in the sub-G1 phase when compared with cultured wild-type astrocytes. We conclude that PrP^C in solution acts as a radical scavenger, and in astrocytes, it is essential for protection from oxidative stress caused by an external chemical agent, which is a likely condition in human neurodegenerative CNS disorders and pathological conditions such as ischemia.

1. Introduction

The cellular prion protein PrP^C is a membrane glycoprotein that is expressed in all cell types, although its expression is consistently higher in the nervous system cells [1–3]. The variety of functions that have been proposed for PrP^C include oxidative stress [4]; immune modulation [5]; differentiation [6]; translocation of metals, such as copper [7]; alteration of copper, zinc and iron homeostasis in the brain [8]; cell adhesion; and transmembrane signaling [3]. The N-terminus PrP^C is a region with four or five repeats of the amino acid sequence PHGGGWGQ, the octapeptide repeat region, which is known to complex with copper ions in its structure [9–12]. Copper(II) likely binds outside of this octapeptide repeat domain [13]. The ability to form this complex is also linked to pH and the concentration of metals in the medium [14,15]. At low metal concentrations, the protein forms a "wraparound" cover by linking several histidines in its sequence. As the pH is related to the metal content at physiological concentrations, up to four copper ions can bind the deprotonated nitrogen atoms of imidazole groups from histidines in the HGGG regions of the octapeptide repeat domain [16], and two copper ions are associated to histidines 96 and 111 in the peptide sequence [17].

Copper is a micronutrient essential for cell survival because it functions as a cofactor for several metalloenzymes. In relation to PrP^C, the octapeptide repeat domain can trap 4 or 5 copper(II) ions, which can alter the redox potential of copper [14]. In fact, copper has two redox states, copper(I) and copper(II); it efficiently catalyzes redox cycles in the presence of endogenous oxygen and hydrogen peroxide, and it generates partially reduced and highly reactive O_2 derivatives, which are known as reactive oxygen species (ROS) [18]. The *in vivo* antioxidant activities of PrP^C have been the subject of intense debate [19–26]. In fact, it has been established that PrP^C generates a positive response in cells to oxidative stress. Brown and co-workers have demonstrated that $Prnp^{0/0}$ mice have a specific cellular phenotype that leads to increased vulnerability to oxidative stress associated with a decrease in superoxide dismutase activity in neurons and astrocytes [27].

PrP^C function has been largely studied in neurons, and the importance of PrP^C expression in astrocytes has only recently emerged. Neurons are particularly sensitive to hydrogen peroxide (H_2O_2), but the neurotoxic effect of H_2O_2 on neurons co-cultured with astrocytes is strongly attenuated; the protective effect of astrocytes depends on the ratio of astrocytes to neurons. Consequently, astrocytes are believed to delay neuronal death in pathological situations in which H_2O_2 has been, at least partially,

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demonstrated to be involved [28]. It follows that the antioxidant activity that protects the neuronal cells from hydrogen peroxide resides in astrocytes. Notably, it has been demonstrated that wild-type astrocytes for PrP^C secrete certain factors, including PrP^C, that are essential for neuronal survival in both wild type and PrP^C-null cells [29].

The objective of this work is to evaluate the protective role of PrP^C against and ROS generation and oxidative stress from hydrogen peroxide in the cell cycle in a new astrocyte cell line derived from PrP^C knockout mice and the wild-type counterpart, which validates the antioxidant activity of PrP^C in solution.

2. Methods

2.1. Chemicals

Reagents were purchased from Sigma–Aldrich, Merck, Invitrogen, Millipore and Fisher Scientific. Solutions were prepared with distilled water purified using a Millipore Milli-Q system (Millipore, Bedford, MA, USA). The reagent 5,5-dimethyl-1-pirroline-*N*-oxide (DMPO, from Sigma) was previously purified as recommended [30].

2.2. Mouse PrP^C expression and isolation

The expression vector containing the cDNA fragment that encodes amino acids 23–231 from the mouse PrP^c protein cloned into the pRSET (Invitrogen™) *Bam*HI–*Eco*RI restriction sites was kindly provided by Ralph Zahn (Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, Switzerland). Expression, purification and elution of His_6-PrP^c were performed as previously described [31]. PrP^Cdel[51-91] was obtained as previously described [32].

2.3. EPR (electron paramagnetic resonance) spin trapping experiments with PrP^C in solution

The EPR spectra were recorded using a Bruker EMX instrument operating at X-ray frequency (9.32 GHz; modulation frequency, 100 kHz; power, 20 mW). In the spin trapping EPR experiments, purified DMPO (Sigma–Aldrich) was used as a spin scavenger. The EPR spectra were generated from a mixture containing an oxidant system that generates ROS (15 μM copper(II)sulfate/3 mM hydrogen peroxide/15 μM ascorbate/phosphate buffer solution, pH 7.4) with and without purified mouse wt PrP^C (3.5 μM). Protein concentrations from 2 to 100 μM were tested, and the difference in oxidation compared with the control experiment (without PrP^C) was demonstrated by the generation of between 45% and 70% less [DMPO-OH] adduct. The concentration of 3.5 μM generated the greatest reduction in adduct formation (70% less). Estimations for DMPO-OH adduct generation were obtained by double integration using the WinEPR Program (Bruker) and a TEMPOL (4-hydroxy-TEMPO, where TEMPO is 2,2,6,6-tetramethyl-1-pipe-ridinyloxy; Sigma–Aldrich) solution as the standard for calibration.

2.4. Cultured astrocytes from PrP^C knockout and wild-type mice

We worked with a line of PrP^C-null animals, which are designated in this study as $Prnp^{0/0}$ (ZrchI) [33], and its wild-type control, $Prnp^{+/+}$ (ZrchI).

The astrocyte primary cultures were prepared as previously described [34] from the cerebral hemispheres of embryonic day 17 (E17) wild type and PrP^C-knockout mice. Briefly, single cell suspensions were obtained by dissociating cells from cerebral hemispheres in Dulbecco's minimum essential medium (DMEM) supplemented with glucose (33 mM), glutamine (2 mM), penicillin/streptomycin (100 IU/100 μg/ml), and sodium bicarbonate (3 mM). The cells were added to plates pre-coated with poly-L-lysine (5 μg/ml) and grown in DMEM enriched with 10% fetal calf serum (FCS). The cultures were incubated at 37 °C in a humidified 5% CO_2-95% air atmosphere, and the medium was changed every 2 days until the cells were 90% confluent.

The immortalized astrocyte cell lines generated in this study were maintained in DMEM supplemented with glucose (33 mM), glutamine (2 mM), penicillin/streptomycin and sodium bicarbonate (3 mM). The primary astrocytes were immortalized based on the protocol described by Morikawa with slight modifications [35]. The cells were transfected with the plasmid pSV3-neo (ATCC), which encodes the SV40 T antigen early regions and neomycin resistance, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Two days after transfection, G418 was added to the astrocytes and then gradually increased over 3 weeks until all of the non-transfected cells had died. Hence, the cells were maintained for two weeks at the highest G418 concentration, 2.08 mg/ml for $Prnp^{0/0}$ and 0.96 mg/ml for $Prnp^{+/+}$. The immortalized cells were tested for over 40 passages. The immortalized astrocytes are referred to as WKO and WWT and were derived from $Prnp^{0/0}$ and $Prnp^{+/+}$ animals, respectively. The cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM, from Sigma–Aldrich) supplemented with 10% fetal bovine serum (from Gibco), 100 U/ml penicillin and 10.0 μg/ml streptomycin (from Sigma–Aldrich). Both the WWT and WKO cells were routinely re-plated at a density of 4×10^4 cells/cm^2.

2.5. Fluorescence measurements

The WWT and WKO cells were plated in a 96 well-plate at a density of 4×10^4 cells/cm^2 to generate monolayers and incubated with hydrogen peroxide (3.0 mM) for 3, 6 and 24 h under the conditions described above. Following incubation, the cells were washed three times with PBS (137 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer at pH 7.4) and treated with a 50.0 μM solution comprising the oxidation-sensitive non-fluorescent probe 2′,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA; Sigma–Aldrich). Following an incubation at 37 °C for 45 min [36,37], the cells were washed three times with PBS, and the levels of intracellular fluorescence were determined immediately using a Varian Cary Eclipse Fluorescence Spectrophotometer at 525 nm. The assays were conducted in quintuplicate.

2.6. Flow cytometry to determine cell cycle progression

Analysis of cell cycle progression was performed according to Nicoletti et al. [38]. Briefly, the cells were stained with 5.0 μg/ml of propidium iodide and analyzed using a CellLab Quanta™ Flow Cytometer from Beckman-Counter™. Adherent WWT and WKO cells were plated at 4×10^4 cells/cm^2 in a 6-well plate, and after 24 h, they were treated with a 1.0 mM hydrogen peroxide solution for the indicated time, trypsinized, combined with detached cells, washed with PBS and stained; the samples were then counted by cytofluorimetric analyses. The experiments were repeated at least five times, and the results displayed reproducibility. The data were analyzed using the WinMDI 2.8 software.

2.7. Statistical analyses

All experiments were repeated at least five times (unless otherwise stated), and the data are expressed as the mean values and standard deviation. The differences between means were assessed using one-way ANOVA with Bonferroni's post-test; p values <0.05 were considered significant.

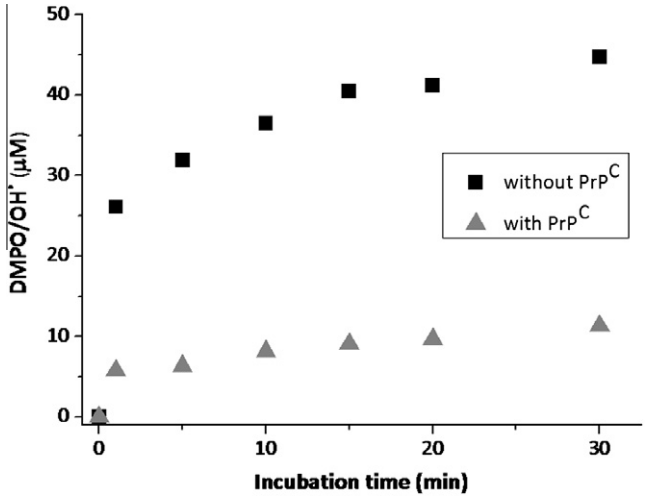

Fig. 1. Isolated PrP^C reduces the rate of ROS generation in an oxidant mixture. A purified DMPO solution (80 mM) was used as spin trap. The spectra were measured after and during the reaction using a gain of 2.00×10^4, a modulation amplitude of 1 G, a time constant of 40.96 ms. The [DMPO-OH] was estimated by double integration using TEMPOL (1–100 μM) as the standard. The graph indicates the mean from three independent sets of experiments.

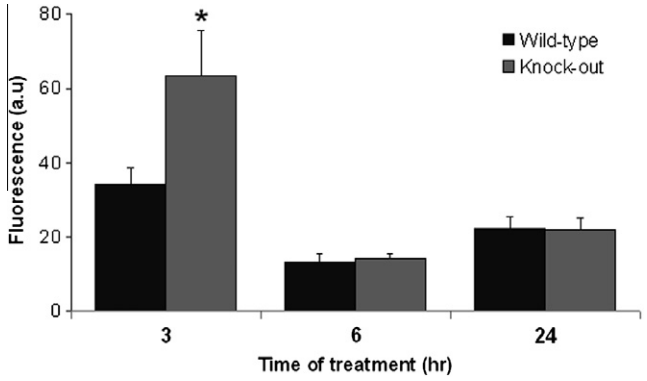

Fig. 2. The effects of hydrogen peroxide treatment on ROS generation in astrocytes: PrP^C knockout cells generate more intracellular ROS. WKO and WWT cells were treated with H_2O_2 at 3 mM for 3, 6 and 24 h. The cells were then incubated with 50 μM DCFH-DA for 30 min at 37 °C and washed 3 times with PBS. The reported fluorescence (absolute units) was measured at 525 nm in a fluorescence spectrophotometer. The assays were conducted in quintuplicate for both cultured cell types, WWT and WKO. The data are the mean values ± standard deviations ($n = 5$). The significant differences between the WWT (wt for PrP^C) and WKO cells (knockout for PrP^C) are indicated by asterisks ($p < 0.05$ (*)).

3. Results

3.1. The PrP^C protein decreases free radical generation in solution

The cellular prion protein is known to reduce oxidative stress in cells; PrP^C-deficient cells exhibit a high Mn-Superoxide dismutase activity, which results in reduced cell defenses [27]. Thereafter, based on these findings, discordant studies were performed to establish whether the prion protein has an SOD1-like activity [39]. On one hand, Brown and colleagues claimed to have found an SOD1-like activity for PrP^C in experiments where the concentration of PrP^C reached 10 mg/ml. On the other hand, using several conditions and different proteins, Jones et al. [40] showed that PrP^C displayed SOD1-like activity, but this activity was low and appeared to be inherent to virtually any protein. In establishing our model, we extrapolated the values obtained by Brown et al. [27] for similar assays using a range of concentrations, 30–100 mg/ml, of purified mouse wt PrP^C and mouse PrP^Cdel[51-91], and we compared the SOD1-like activity to a positive control, SOD1. We concluded that the SOD-like activity displayed by these proteins is almost negligible compared with SOD1 (data not shown).

Table 1
Percentage of WWT cells in the different cell cycle phases. The control refers to cells without H_2O_2 treatment. The data are presented as the mean values ± standard deviations ($n = 5$).

	Wild-type (%)			
	SubG1	G1	S	G2/M
24 h				
Control	7.5 ± (0.4)	17.9 ± (0.4)	48.9 ± (0.7)	22.5 ± (0.5)
H_2O_2	12.4 ± (0.2)	14.3 ± (0.6)	44.7 ± (1.8)	22.8 ± (1.1)
48 h				
Control	8.0 ± (0.4)	17.5 ± (0.9)	45.2 ± (2.0)	28.0 ± (1.4)
H_2O_2	27.5 ± (1.4)	9.1 ± (0.5)	36.0 ± (1.8)	21.0 ± (1.1)

On the other hand, we observed that the isolated PrP^C protein in solution could decrease the concentration of the DMPO-OH radical adduct generated in an oxidant system (copper(II)/ascorbate/H_2O_2). The generation of radicals in this Fenton mixture, which comprised hydroxyl radicals, was measured through the nitrone DMPO, and the concentration was determined by measuring the area under the peak [41]. The presence of soluble PrP^C in this oxidant solution reduced the concentration of DMPO-OH by 45–70% depending on the protein concentration. The best result was obtained for a 3.0 μM concentration of PrP^C (Fig. 1) with 70% less DMPO-OH trapped and measured in the system.

3.2. PrP^C is necessary for astrocytes in culture to reduce ROS generation under oxidative stress

WKO, which is an astrocyte cell line derived from PrP^C knockout mice, and WWT, the wild type counterpart, have been used to evaluate the resistance of glia cells without PrP^C to ROS generation and oxidative stress caused by exogenous hydrogen peroxide. We have quantified the level of intracellular ROS formed after the cells were exposed to H_2O_2 using the membrane-permeable non-fluorescent cell probe DCFH-DA, which has a highly fluorescent oxidized form (2′,7′-dichlorofluorescein; DCF) [42,43]. After 24 h of treatment with H_2O_2, the untreated cells had a weak fluorescence intensity, which varied little with respect to time (data not shown). In contrast, incubation with an internal ROS source resulted in an early and rapid increase in fluorescence only in WKO cells (Fig. 2) for three hours before the fluorescence returned to the levels observed for WWT cells. Thus, WKO cells generated a swift increase, which could correspond to a protective role for PrP^C, and the decrease in fluorescence intensity observed later resulted from compensatory mechanisms.

Table 2
Percentage of WKO cells in the different cell cycle phases. The control refers to cells without H_2O_2 treatment. The data are presented as the mean values ± standard deviations ($n = 5$).

	Knock-out (%)			
	SubG1	G1	S	G2/M
24 h				
Control	6.3 ± (0.1)	6.0 ± (0.3)	57.5 ± (0.2)	28.7 ± (1.2)
H_2O_2	20.8 ± (0.9)	4.1 ± (0.2)	39.8 ± (2.0)	27.3 ± (1.4)
48 h				
Control	20.7 ± (0.8)	5.5 ± (0.3)	39.1 ± (1.1)	32.3 ± (1.2)
H_2O_2	54.1 ± (2.7)	33.8 ± (1.7)*		

* Differentiation of phase was not observed in this case.

3.3. PrP^C is important for astrocyte viability in culture under oxidative stress conditions

Cell cycle measurements can be a useful tool to verify the influence of oxidant treatment in cell culture [44]. The modality of cytotoxic effects caused by hydrogen peroxide in cultured astrocytes was assessed by cytofluorimetric analyses after staining the cell DNA with the fluorochrome propidium iodide. As expected and shown in Fig. 3A, 48 h of treatment with H_2O_2 promoted WWT cell progression towards the sub-G1 (apoptotic) phase compared with

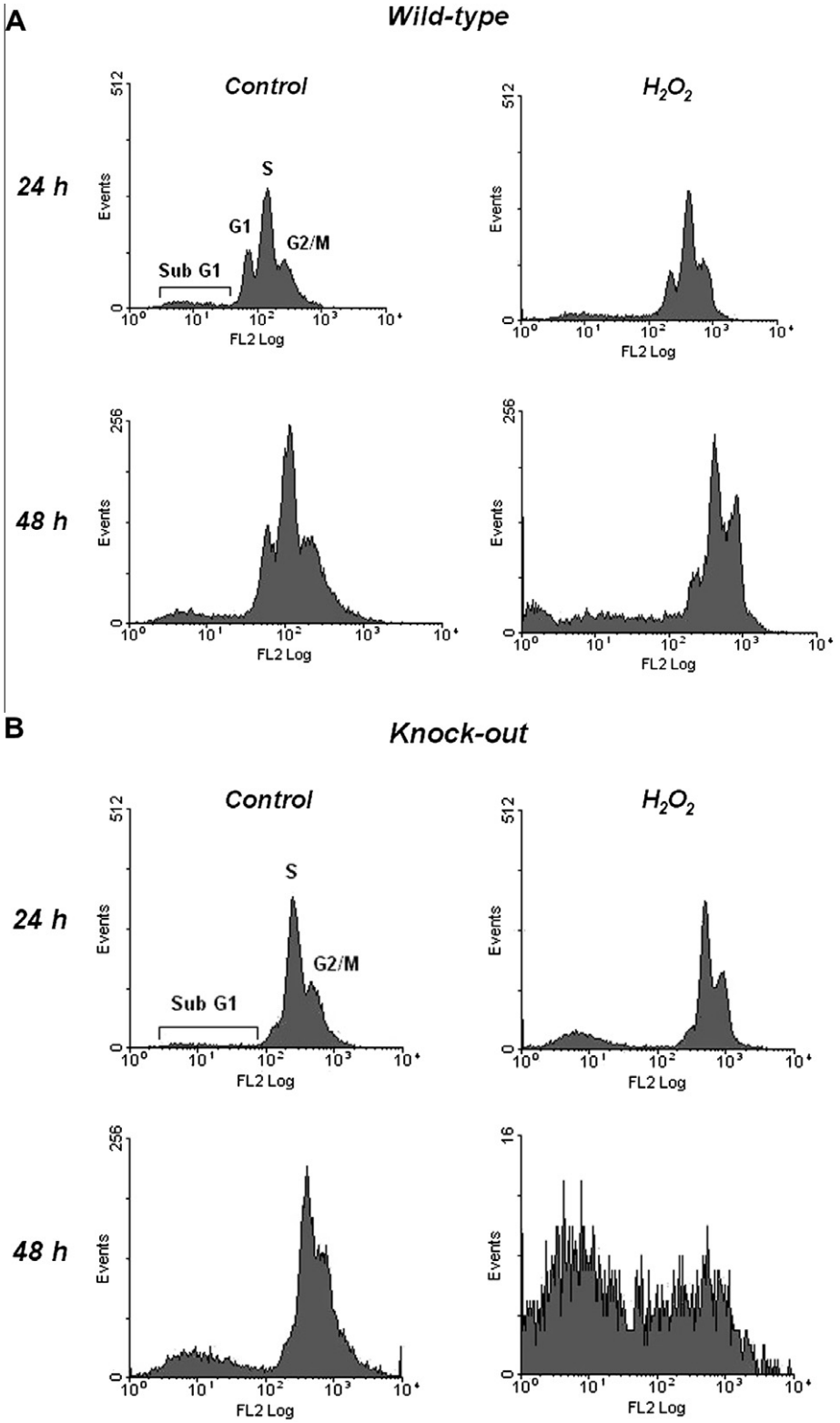

Fig. 3. The effects of hydrogen peroxide treatment on the astrocyte cell cycle: PrP^C knockout cells (WKO) are less resistant and had a higher percentage of cells in the sub-G1 phase. Both WKO and WWT were treated with 1 mM H_2O_2 for 24 and 48 h. The cells were then treated with propidium iodide to determine cell cycle progression and apoptosis through cytofluorimetric analyses. The reported cell cycle plots and the percentage of nuclei in the different phases are from a typical experiment performed 5 times that produced similar results. (A) WWT; (B) WKO. The control refers to cells without H_2O_2 treatment.

the control cells without the oxidant treatment (see Table 1). However, in WKO cells, the intense cell cycle arrest was followed by an increased percentage of the sub-G1 population after treatment for 24 h, and after 48 h more than a half of the population was in the sub-G1 phase (see Table 2). The low population observed in the flow cytometry was due to the high mortality of the WWO cells under oxidant treatment. Even without treatment PrP^C knockout astrocytes, shown a higher population in the sub-G1 phase (Fig. 3B) compared with the wt astrocytes (Fig. 3A). Moreover, no significant cell cycle arrest in the G1 or G2/M phases was observed during the treatment of these cells (see Tables 1 and 2).

4. Discussion

The antioxidant activity of the prion protein has been highly discussed in the literature, especially with reference to its potential superoxide dismutase-like activity [39,45]. The source for this interest is likely that PrP^C binds 4 or 5 copper ions in its structure, and as copper is a redox-active metal ion, it is probable that PrP^C participates in a redox process as a ROS scavenger or a general antioxidant protein. Herein, we quantified the ROS scavenger activity of PrP^C *in vitro* and demonstrated its importance for astrocyte survival in an oxidative environment.

Free radical generation in solution, which includes hydroxyl radicals, can be measured by the nitrone DMPO in a competitive manner compared with other redox sensitive species in the medium. We observed that PrP^C in the oxidant medium is associated with an approximately 70% decrease in the radical adduct DMPO/$OH^{\cdot}$ concentration. Similarly, we demonstrated that PrP^C in solution can act as a hydroxyl radical scavenger and prevent the radicals from reacting with the trap DMPO.

In cultured astrocytes generated from wild type and PrP^C knockout mice, an oxidant medium was added, and both ROS scavenger activity and cell cycle progression were examined under these conditions. The concept that redox cycling controls the mammalian cell cycle through modulation of intracellular antioxidant/oxidant species has received much consideration in the literature [46]. In this study, the absence of PrP^C caused abnormal elevations in ROS accumulation in the astrocytes over the first 3 h of treatment. Moreover, we linked this increase in ROS levels to strong alterations in the cell cycle, including an increase in the sub-G1 phase cells after 24 and 48 h of oxidant treatment.

As expected, H_2O_2, which is a strong ROS generator, promotes apoptosis in cells after 24 h, and this effect is stronger at 48 h but only when PrP^C is absent in astrocytes. In wild type cells, the oxidant treatment increased the amount of apoptotic cells after 48 h and the proportion of cells in the sub-G1 phase by 27%, which was much less than in the PrP^C knockout cells. These results suggest that PrP^C is important for reducing hydrogen peroxide toxicity in astrocytes, which prevents the cell death independently of an SOD-like activity. This finding raises the following questions: how does PrP^C mediates such activity, if other partners of this modulating protein are involved, and importantly, what is the importance of such anti-redox activity in the context of neurodegenerative diseases.

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