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Expression of Prion Protein Increases Cellular Copper Binding and Antioxidant Enzyme Activities but Not Copper Delivery*

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The N-terminal region of the prion protein PrP^C contains a series of octapeptide repeats. This region has been implicated in the binding of divalent metal ions, particularly copper. PrP^C has been suggested to be involved in copper transport and metabolism and in cell defense mechanisms against oxidative insult, possibly through the regulation of the intracellular CuZn superoxide dismutase activity (CuZn-SOD) or a SOD-like activity of PrP^C itself. However, up to now the link between PrP^C expression and copper metabolism or SOD activity has still to be formally established; particularly because conflicting results have been obtained *in vivo*. In this study, we report a link between PrP^C, copper binding, and resistance to oxidative stress. Radioactive copper (^{64}Cu) was used at a physiological concentration to demonstrate that binding of copper to the outer plasma cell membrane is related to the level of PrP^C expression in a cell line expressing a doxycycline-inducible murine PrP^C gene. Cellular PIPLC pretreatment indicated that PrP^C was not involved in copper delivery at physiological concentrations. We also demonstrated that murine PrP^C expression increases several antioxidant enzyme activities and glutathione levels. Prion protein may be a stress sensor sensitive to copper and able to initiate, following copper binding, a signal transduction process acting on the antioxidant systems to improve cell defenses.

Prion diseases form a group of fatal neurodegenerative disorders including Creutzfeldt-Jakob diseases, Gerstmann-Sträussler Syndrome, Kuru and Fatal Familial Insomnia in humans, and scrapie and bovine spongiform encephalopathy in animals (1). All these disorders are characterized by the accumulation of an abnormally folded isoform of the cellular prion protein PrP^C,[1] denoted PrP^{Sc}, which represents the major component of infectious prion diseases (2). The formation of PrP^{Sc} from PrP^C is accompanied by profound changes in structure and biochemical properties. PrP^C rich in α-helical regions is converted into a molecule with highly β-sheeted structures and partial resistance to proteolytic digestion (2, 3). The conversion of PrP^C into PrP^{Sc} remains enigmatic. Biosynthesis of PrP^C is necessary for PrP^{Sc} formation, as mice lacking PrP^C are resistant to scrapie infection (4).

Human PrP^C has 253 amino acids and is mainly expressed on neurons (5, 6). In its N-terminal region, a repeated sequence of five octapeptides can be found, which was shown to bind copper and zinc (7–9). The protein may have some superoxide dismutase-like activity and therefore a possible protective function against oxidative stress (10). Wild-type mouse brains have a significantly higher level of membrane-associated copper than PrP^C-deficient mice. Treatment with phosphatidylinositol phospholipase C (PIPLC) specifically reduced the copper content from wild type mice but had no effect on the copper content of PrP^C knockout mice (8). However, these results have not been confirmed (11). Incorporation of radioactive-labeled copper into CuZn-SOD was found to be proportional to the level of PrP expression (12). Pattison and Jebbett (13) noticed more than 30 years ago the similarity between prion histopathology to the histopathology induced by a copper chelator, cuprizone. The incidence of chronic Wasting disease (CWD), a sporadic prion disease of deer and elk, was observed to be higher in regions where the soil had a low copper content (14). Therefore, prion diseases may be related to an alteration of copper transport and a loss of copper-enzyme activities.

In a previous work, we demonstrated that neuronal cells infected with prion strains resulted in an alteration of the molecular mechanism promoting cellular resistance to ROS (15). The same alteration of antioxidant enzymes was shown in infected animals (16, 17). In the present study, we used a transfected transgenic cell line with a doxycycline-inducible murine PrP^C gene to investigate the involvement of PrP^C in copper metabolism and in the resistance mechanism to toxic stress.

EXPERIMENTAL PROCEDURES

Cell Culture and Construction—Murine PrP^C was cloned in the pTRE plasmid (Clontech), and the resulting plasmid was transfected by the LipofectAMINE method (Invitrogen) into rabbit kidney epithelial cells (RK13) (18, 19). Stable transfectants were selected in the presence of puromycin (1 μg/ml), and one (A74) was amplified for further study. RK13 and A74 cells were grown at 37 °C in a 5% CO_2-enriched atmo-

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[1] The abbreviations used are: PrP^C, cellular isoform of prion protein; SOD, superoxide dismutase; dox, doxycycline; PIPLC, phosphoinositol phospholipase C; ROS, reactive oxygen species; GPX, glutathione peroxidase; GR, glutathione reductase; MDA, malondialdehyde acid; SIN-1, 3-morpholinosydnonimine; PrP^{Sc}, scrapie isoform of prion protein; MTT, (4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; TBARS, thiobarbituric acid reactants.

sphere in minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and were usually split at one-fourth dilution each week.

Immunofluorescence and Western Blot Analysis—Immunofluorescence analysis on living A74 cells was performed at 4 °C, with rabbit polyclonal antibody P45–66, raised against synthetic peptide encompassing mouse PrP^C (MoPrP) residues 45–66. Fluorescein-conjugated IgG was used as second antibody.

For Western blot analysis, confluent cells were washed twice with cold phosphate-buffered saline, calcium- and magnesium-free, and lysed for 30 min at 4 °C in Triton-deoxycholate lysis buffer (1× buffer is 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 50 mM Tris-HCl, pH 7.5) plus protease inhibitors. After 1 min of centrifugation at 10,000 × *g*, the supernatant was collected, and its protein concentration was measured by the BCA assay (Pierce). The equivalent of 20 μg of total protein in SDS loading buffer was subjected to 12% SDS-PAGE electrophoresis followed by electroblotting on polyvinylidene difluoride in Tris-glycine buffer containing 20% methanol. The membrane was blocked with 5% nonfat dry milk in TBST (0.1% Tween 20, 100 mM NaCl, 10 mM Tris-HCL, pH 7.8) for 1 h at room temperature, and MoPrP was detected by immunoblotting with P45–66 antibody as previously described (20). After adding the second antibody (horseradish peroxidase-coupled rabbit IgG), immunoreactive proteins were detected with ECL Western blot system. Quantification was achieved by densitometric scanning.

PrP^C analysis in culture medium was immunodetected by Western blot. To release cell surface PrP^C, cultures were treated with PIPLC (0.2 units/ml) in opti-MEM serum-free medium at 37 °C for 2 h. Proteins were precipitated from the PIPLC incubation medium with at least 4 volumes of methanol at −20 °C, collected by centrifugation, and immunoblotted with P45–66 antibody using ECL visualization.

Cellular ^{64}Cu Binding—Cells (RK13 and A74) were cultured in 35-mm Petri dishes. Culture medium was replaced by 2 ml of fresh complete medium containing different concentrations of dox (0–500 ng/ml) to stimulate murine PrP^C expression in A74 cells, and 1.6 μM ^{64}Cu (CIS biointernational, Gif-sur-Yvette, France; specific activity 20 mCi/mg) to evaluate copper binding to cells as a function of the level of murine PrP^C expression. Non-transfected RK13 cells were used as control and treated under the same conditions. Cells were incubated at 37 °C under 5% CO_2. The radioactive medium was removed after 30–40 min, 2, 4, 8, 10, 24, and 26 h. Cells were rinsed twice with 2 ml of diluted Puck's saline A solution (Invitrogen), and harvested after addition of 1 ml of 0.25% trypsin. After harvesting, each dish was rinsed with 1 ml of Puck's saline A solution. The final 2 ml obtained for each dish were counted for 2 min using a Packard Cobra III, mono well gamma counter (Packard Instrument Company, Meriden, CT). Protein content was assayed with the BCA protein assay reagent kit. Data were analyzed using a "self made" computer half-life calculation program, to obtain results as μCi of ^{64}Cu incorporated or retained per mg of protein.

Cellular Copper Determination—Stimulated (500 ng/ml dox for 24 h) or unstimulated A74 cells were cultured in the presence or absence of 100 μM $CuSO_4$ for 1 h. For intracellular copper determination, cells were trypsinized, washed three times in Ca/Mg-free phosphate-buffered saline, and lysed by three cycles of freeze-thawing. Lysates (total extract) were then centrifuged at 13,000 rpm for 10 min to obtain the soluble fraction. Copper concentration was determined by electrothermal atomic absorption spectrophotometry (PerkinElmer Life Sciences). Their levels were normalized to the protein content, measured with a protein assay kit.

Cell Viability Assay—Cell viability was determined by a modified 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (21). Briefly 3000 cells per well were plated in 96-well microtiter plates with 100 μl of complete medium. The next day, the medium was changed, and the cells were challenged for 24 h with different drugs. The medium was then changed, and the cells were incubated for an additional 24 h without drugs. For the MTT assay, 10 μl of MTT (5 mg/ml stock in phosphate-buffered saline) were added to each well for 3 h at 37 °C. 100 μl of dimethyl sulfoxide (Me_2SO) were added to dissolve the formazan crystals, and plates were shaken for 5 min on a plate shaker to ensure adequate solubilization. The absorbance readings for each well were performed at 570 nm using Multiscan ascent plate reader (Labsystems). The absorbance is proportional to viable cell number, and survival was calculated as the percentage of the staining values of untreated cultures.

Lipid Peroxidation—Lipid peroxidation was evaluated using an assay based on fluorescence of thiobarbituric acid reactants measured after extraction with *n*-butyl alcohol (22). Subconfluent cells were trypsinized in 75-cm^2 flasks, washed three times by 10 ml of isotonic, trace element-free Tris-HCl buffer (400 mM, pH 7.3), and then lysed in hypotonic Tris-HCl buffer (20 mM) by five freeze-defrost cycles. 750 μl of a mixture of thiobarbituric acid at 8 g/7% perchloric acid (2:1) were added to a 100-μl sample. After agitation the mixture was placed in a 95 °C water bath for 60 min and then cooled in an ice bath. The fluorescent compound was extracted by mixing with *n*-butyl alcohol for 2 min. After centrifugation the fluorescence in the *n*-butyl alchohol phase was determined with an Aminco-Bowman fluorimeter (PerkinElmer Life Sciences) with excitation at 532 nm and emission at 553 nm. A blank was run for each sample. The calibration curve was created with a stock solution of 1,1,3,3-tetraethoxypropane prepared in alcohol. The results were expressed as TBARS, μmol/g of protein.

Superoxide Dismutase Activity—For SOD activity, subconfluent cells in 75-cm^2 flasks, were washed three times and collected in 10 ml of isotonic, trace element-free Tris-HCl buffer (400 mM, pH 7.3), and lysed in hypotonic Tris-HCl buffer (20 mM) by five freeze-defrost cycles. After 10 min of centrifugation at 4000 rpm, 4 °C, the lysate was assayed for metalloenzyme activities and soluble protein content. Total SOD, Mn-SOD, and CuZn-SOD were determined using the pyrogallol assay following the procedure described by Marklund and Marklund (23), based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD, and spectrophotometrically read at 420 nm. Briefly, 50 μl of the sample were added to 1870 μl of Tris (50 mM)-DTPA (1 mM)-cacodylic acid buffer, pH 8.3 and to 80 μl of pyrogallol (10 mM) in order to induce an absorbance change of 0.02 in the absence of SOD. The amount of SOD inhibiting the reaction rate by 50% in the given assay conditions was defined as one SOD unit. The specific CuZn-SOD inhibition by KCN (60 μl of KCN, 54 mM) added to 300 μl of lysate allowed the Mn-SOD determination under the same conditions. Each sample was assayed twice, and results were expressed as SOD units and normalized to the cell protein content.

Analysis of Glutathione-dependent Antioxidant System—For the determination of total glutathione levels, confluent cells in 25-cm^2 flasks were washed three times in phosphate-buffered saline and collected in 10 ml of isotonic, trace element-free Tris-HCl buffer (400 mM, pH 7.3), and lysed in hypotonic Tris-HCl buffer (20 mM) by five freeze-defrost cycles. Samples of whole lysate were deproteinized by adding metaphosphoric acid (6%) (lysate-metaphosphoric 5:1, v/v). After 10 min at 4 °C the solutions were spun at 4000 rpm for 10 °C at 4 °C, and the supernatants were assayed for total glutathione content according to the Akerboom and Sies method (24).

The glutathione peroxidase (GPX) activity was assayed by the method of Gunzler *et al.* (25). GPX was measured in a coupled reaction with glutathione reductase (GR), using *tert*-butylhydroperoxide as substrate. Briefly, 25 μl of the sample were added to 900 μl of Tris (50 mM), EDTA, sodium azide buffer, pH 7.6 (azide was included in the assay mixture to inhibit interference of catalase) and 20 μl of glutathione (0.15 M), 20 μl of glutathione reductase (200 units/ml), 20 μl of $NADPH_2$ (8.4 mM) in order and incubated for 1 min for mixture equilibrium. Then 20 μl of *tert*-butylhydroperoxide were added, and the decrease of the absorbance was monitored at 340 nm for 200 s. The difference in absorbance per minute was used to calculate the enzyme activity, and results were expressed as GPX units/g of protein.

GR activity was determined by following the oxidation of NADPH to NADP+ during the reduction of oxidized glutathione (GSSG) (26). The main reagent was prepared by combining 18 ml of KH_2PO_4 (139 mM), EDTA (0.76 mM), pH 7.4, and 2 ml of $NADPH_2$ (2.5 mM). 20 μl of sample were added with 220 μl of the main reagent, and then 30 μl of GSSG (22 mM) plus 10 μl of KH_2PO_4 were added to start the reaction; the absorbance was followed at 340 nm for 175 s. The difference in absorbance per min was used to calculate the activity of the enzyme. The results were expressed as glutathione reductase units/g of protein.

RESULTS

Doxycycline-inducible Expression of Murine PrP in A74 Cells—We used the tetracycline-inducible (tet-on) system (18, 27) to achieve regulated high-level expression of the murine PrP^C. After transfection of several cell lines, a strong inducible expression of murine PrP^C was obtained in most of the clones derived from a rabbit kidney epithelial cell line (RK13). Data obtained with a representative clone (A74) are presented in this article. Expression of murine PrP^C was related to dox concentration in the culture medium, detectable at 10 ng/ml dox and reaching a maximum at 500 ng/ml of dox (Fig. 1). No PrP^C was detected in either unstimulated A74 cells (Fig. 1) or non-transfected RK13 (data not shown), confirming that ex-

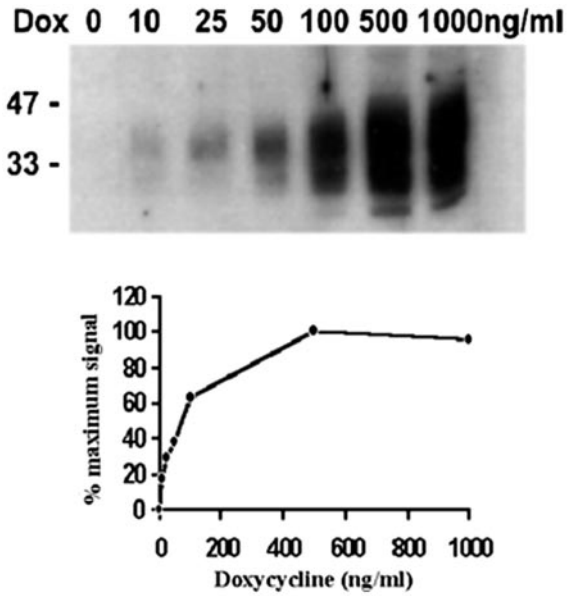

FIG. 1. **PrP^C induction is doxycycline-dependent.** Dox was added at different concentrations (0, 10, 25, 50, 100, 500, and 1000 ng/ml) to medium for 24 h, and PrP^C expression was determined in A74 cells by Western blot. The equivalent of 20 μg of protein (as determined with the BCA protein assay kit) were loaded to a 12% polyacrylamide gel, transferred onto polyvinylidene difluoride membrane, and PrP^C was detected with antibody P45–66 raised against the N terminus of the protein. PrP^C expression reaches a maximum at 500 ng/ml of dox; after this concentration we have a plateau. Specific murine PrP^C bands were quantified by densitometry and plotted as a percentage of maximum signal of PrP^C expression in A74 cells. Molecular mass markers are indicated on the *left* in kDa.

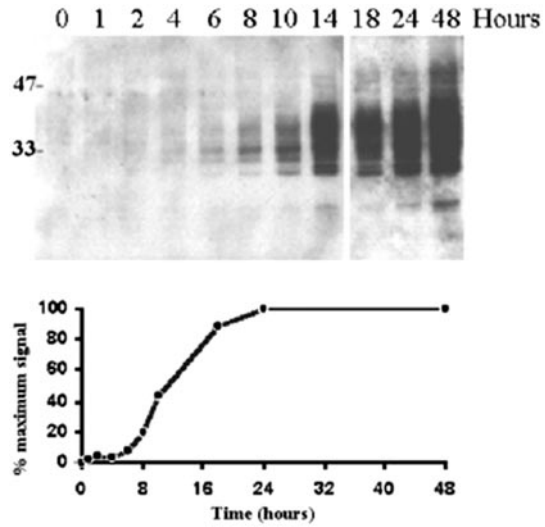

FIG. 2. **Time course of PrP^C expression in A74-stimulated cells.** 500 ng/ml of dox was added at time 0, and cells were harvested and lysed at different times (0, 1, 2, 4, 6, 8, 10, 24, and 48 h). 20 μg of protein were Western blotted and analyzed with the same antibody P45–65. Expression of PrP^C can be detected 8 h after induction with a plateau at 24 h. Specific murine PrP^C bands were quantified by densitometry and plotted as a percentage of maximum signal of PrP^C expression in A74 cells. Molecular mass markers are indicated on the *left* in kDa.

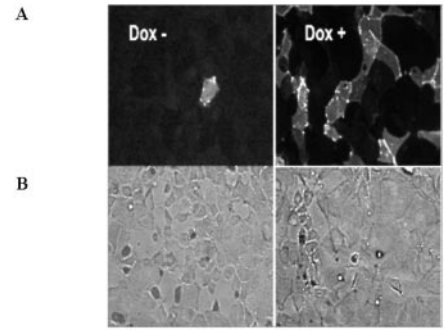

FIG. 3. **A74 cells expressed PrP^C on the cell membrane.** *A*, fluorescence microscopy photography of stimulated (500 ng/ml dox for 24 h) or unstimulated A74 cells. Fluorescein-conjugated IgG were used as second antibody. *B*, phase contrast microscopy for the same cells. 32% of stimulated cells produced PrP^C at the outer side of cell membrane.

pression of endogenous, rabbit PrP was undetectable in these cells (18). We also studied the induction kinetics of PrP^C expression in A74 cells stimulated with 500 ng/ml dox. Expression of PrP^C can be detected 8 h after induction, and a plateau was obtained at 24 h (Fig. 2). Not all, although up to 32% of cells, produced PrP^C at a high level and expressed it on the outer face of the plasma membrane (Fig. 3).

^{64}Cu Binding Is Correlated to PrP^C Expression—No significant difference in copper binding was observed during the first 4 h following induction of PrP^C expression (Fig. 4*A*). This apparent lack of effect might be due to the lack of detectable PrP^C 8 h following addition of dox (Fig. 2). Then, the ^{64}Cu binding increased proportionately to dox concentration. 26 h following the addition of 500 ng/ml of dox, copper binding was 2.7-fold higher in stimulated cells compared with the unstimulated cells. This increase was due to PrP^C expression and not to an effect of dox on copper binding because dox had no effect in non-transfected RK13 control cells (Fig. 4*B*).

The copper binding curve obtained with the non-transfected control cells RK13 was similar to that obtained with unstimulated A74 (Fig. 4, *A* and *B*). This corresponds to the copper uptake via the classical transport systems (CTR1 and other potential transporters), while the increase observed in dox-stimulated A74 corresponds to the binding or transport activity of PrP^C.

To study the influence of PrP^C cleavage on copper binding, the cells were treated for 2 h with 0.2 units/ml PIPLC at 37 °C before measuring copper binding. PIPLC pretreatment dramatically decreases ^{64}Cu binding in stimulated A74 cells (500 ng/ml dox) but had no effect on unstimulated cells (Fig. 4*C*). However, there was a small difference in the kinetics of copper binding between stimulated cells treated with PIPLC and unstimulated cells presumably because PIPLC might not have cleaved all PrP^C from membranes. PIPLC pretreatment had no effect on stimulated or unstimulated non-transfected RK13 cell controls (Fig. 4*D*).

PrP^C Binds Copper at the Outer Side of the Cell Membrane at Physiological Concentrations—To investigate the location of bound copper and check for a real entry, cells were rinsed twice with 2 ml of diluted Puck's saline A solution (Invitrogen) 24 h after PrP^C induction with 500 ng/ml of dox and incubated in a radioactive medium containing ^{64}Cu. Treatment with PIPLC decreased the amount of PrP^C in stimulated cells (Fig. 5*A*) concomitantly with a high decrease in cell-associated radioactivity (Fig. 5*C*). Immunoblotting demonstrated that PrP^C was found only in the medium when stimulated cells (500 ng/ml dox) were treated with PIPLC (Fig. 5*B*). The release of PrP^C was correlated with a high increase of radioactivity in the medium (Fig. 5*D*). These findings indicate that PrP binds copper at the outer side of the cell membrane and that cleavage of PrP^C liberates copper into the medium. The difference in copper binding between stimulated and unstimulated cells was abolished after treatment with PIPLC indicating that no copper had been taken up by the PrP^C. Therefore, PrP^C does not transport copper inside the cell at physiological concentrations. These results are confirmed by measuring cellular copper con-

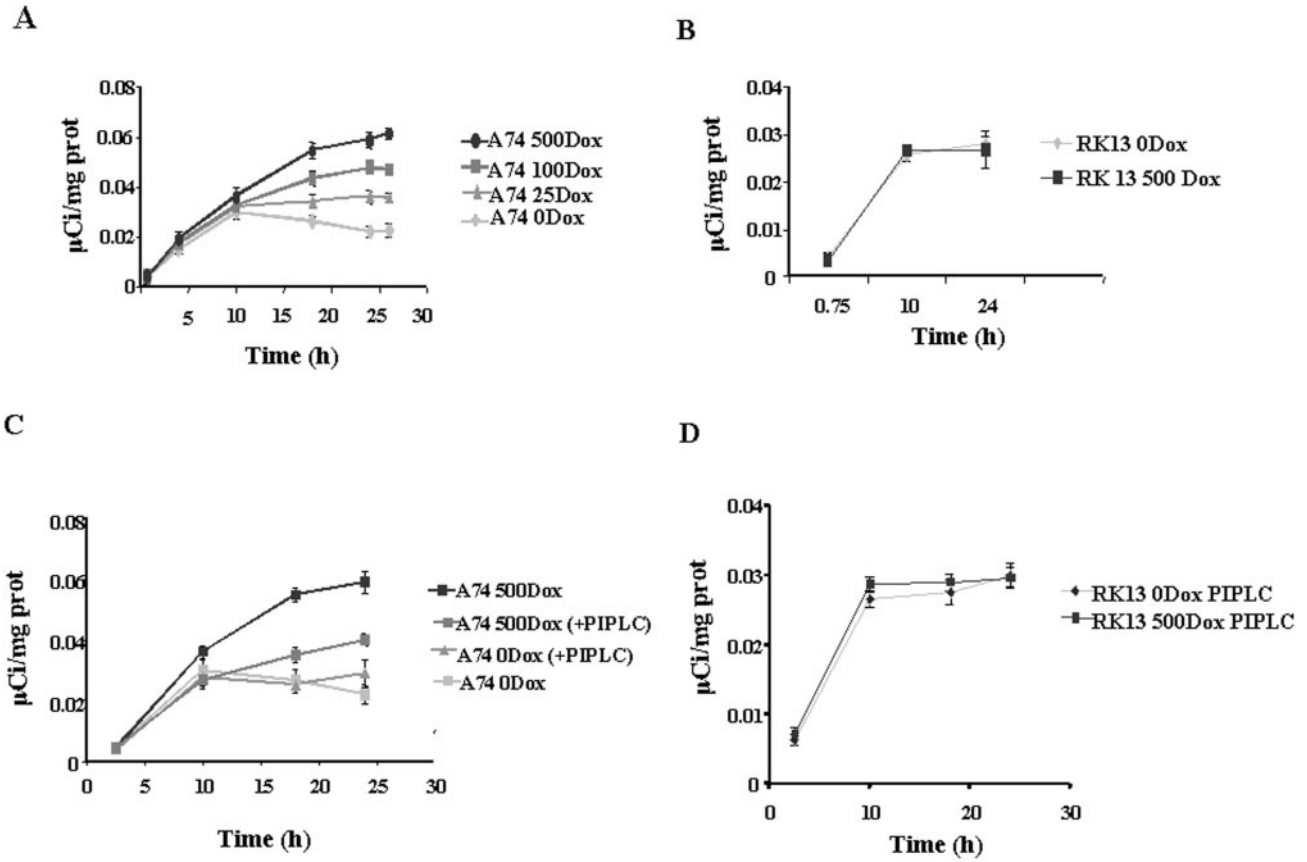

FIG. 4. **Relationship between PrP^C expression and ^{64}Cu binding.** *A*, ^{64}Cu binding increases in proportion to PrP^C expression in A74 cells. 0.1 μg of ^{64}Cu was added to A74 cell culture medium containing different concentrations of dox (0, 25, 100, and 500 ng/ml). Cells were incubated for 0.6, 2, 4, 8, 10, 24, and 26 h in radioactive media and harvested, and ^{64}Cu binding was measured. Copper binding was correlated to murine PrP^C expression. *B*, dox treatment did not change copper binding in non-transfected control cells RK13. 0.1 μg of ^{64}Cu was added to RK13 cell culture medium containing 0 or 500 ng/ml of dox. Cells were incubated for 0.75, 10, and 24 h in radioactive media, harvested, and ^{64}Cu binding was measured and normalized to protein cell lysates. *C*, the experiments described above were repeated, but cells were treated with 0.2 units/ml PIPLC before measuring radioactivity in stimulated and unstimulated A74 cells (*C*) or in stimulated and unstimulated non-transfected RK13 cells (*D*). Controls were at times (2.5, 10, 18, and 24 h). Copper binding was quantitated and normalized to the protein of cell lysates. Data represent the mean of three experiments ± S.D.

tent by electrothermal atomic absorption spectrophotometry. Copper content was increased up to ~2-fold in the total but not the soluble fraction of stimulated cells (see Table II). Thus, at physiological concentrations PrP^C did not transport copper from the extracellular medium to cytoplasm since there was no difference in ^{64}Cu content between the cytosolic fractions of stimulated and unstimulated cells.

Effect of PrP^C Expression on Transition Metal Toxicity—As induction of PrP^C expression did not increase the incorporation of physiological levels of copper, we decided to investigate whether PrP^C-dependent bound copper could play a role in the protection of cells toward copper toxicity. Since it was suggested that manganese can compete with copper for the binding sites (28), the resistance to this metal was also tested. The data presented in Fig. 6, *A* and *B* clearly show that cells overexpressing PrP^C (1000 dox) withstood higher copper (but not manganese doses, Fig. 6*B*) than unstimulated (0 dox) cells or stimulated RK13 cells. This resistance to copper was more related to stimulated cells, with an LC50 of 540 and 341 μM $CuSO_4$ when they were compared with unstimulated cells. Therefore PrP increases cellular resistance to copper but not to manganese toxicity.

PrP^C Overexpression Increases Resistance to Oxidative Stress and Antioxidant Enzyme Activities—Because it has been suggested that one of the physiological functions of PrP^C could be in the protection of cells toward an oxidative stress (10), we investigated both the resistance to an oxidative stress and the activities or levels of the main antioxidant systems in cells overexpressing PrP^C. To study the relationship between prion protein expression and resistance to oxidative stress, MTT assays were performed following 3-morpholinosydnonimine (SIN-1) treatments, which generates different free radicals: $O_2^{\cdot-}$, NO$^{\cdot}$, and/or NO_x, and other potent oxidants such as $ONOO^-$ (46). Stimulated cells (500 ng/ml dox) presented higher resistance (cell viability, 95%) to this oxidative stress when compared with unstimulated cells (0 dox) (cell viability, 59%) or control cells (RK13 0 or 500 dox) (Fig. 7*A*). In contrast, cells overexpressing PrP^C were surprisingly more susceptible to hydrogen peroxide than unstimulated cells. Treatment for 3 h with different concentrations of H_2O_2 induced a more severe decrease in viability for doses exceeding 200 μM (Fig. 7*B*) in stimulated cells as compared with unstimulated cells. At 500 μM H_2O_2-stimulated cells revealed ~50% lower viability than unstimulated cells. These data demonstrate that PrP^C expression decreases resistance to peroxide toxicity.

We also evaluated the involvement of PrP^C expression in the cellular defense against oxidative stress by measuring different antioxidant activities such as SOD, GPX, GR, and glutathione levels. Induction of PrP increases total SOD (~21%), CuZn-SOD (~27%), GR (~64%) activities, and GSH levels (~78%), while the activities of GPX and mitochondrial Mn-SOD remain unchanged (see Table I). Interestingly, Western blot detection of CuZn-SOD in A74 cells indicates that the total level of this protein was unchanged in stimulated and unstimulated cells

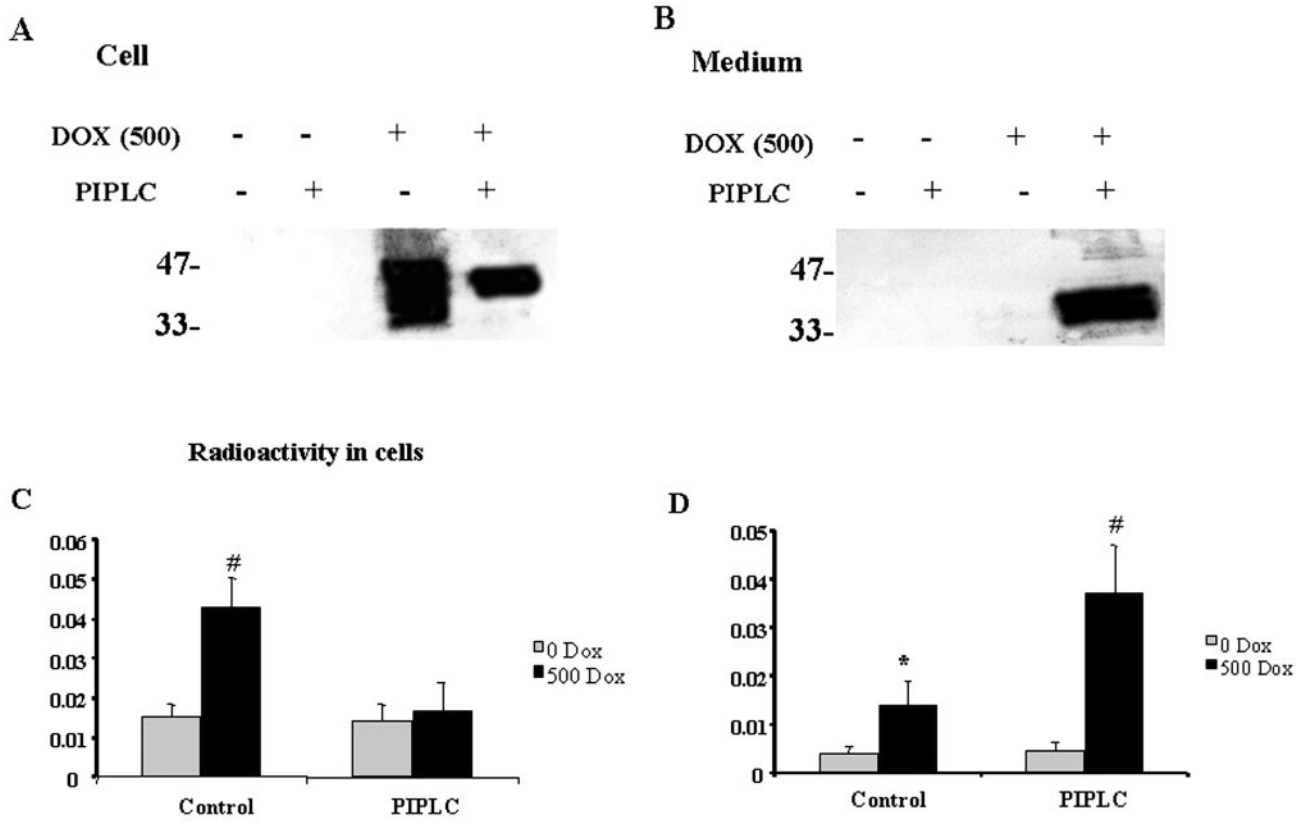

FIG. 5. **Effect of PIPLC treatment on stimulated and unstimulated A74 cells.** *A*, after 24 h of PrP^C induction with 500 ng/ml dox in radioactive media, cells were treated with 0.2 units/ml PIPLC for 2 h at 37 °C, lysed in lysis buffer and loaded onto a 12% polyacrylamide gel, transferred onto polyvinylidene difluoride membrane, and PrP^C was detected with antibody P45-66 raised against the N terminus of the protein. *B*, proteins in culture were precipitated with 4 volumes of methanol, and PrP^C was detected with P45-66 antibody. *C*, 24 h after PrP^C induction with or without 500 ng/ml of dox in a radioactive medium containing ^{64}Cu, cells were treated with PIPLC (0.2 units/ml) for 2 h at 37 °C, and radioactivity was measured. *D*, radioactivity was measured in the opti-MEM medium of the same cells and normalized to protein cell lysates and protein in the media. Results are expressed as the mean of three experiments ± S.D. of radioactivity (μCi of ^{64}Cu/mg of protein). *, $p < 0.01$; #, $p < 0.005$.

(Fig. 8). This may reflect an increase in CuZn-SOD activity of stimulated cells resulting from increased copper incorporation into SOD or from SOD-like activity of PrP-copper complexes. Finally to detect if PrP expression decreases oxidative damage, lipid peroxidation was evaluated by measuring the formation of TBARS as a stress biomarker. The basal level of oxidative damage was significantly lower in stimulated cells as compared with unstimulated and control cells. These data indicate that PrP^C expression increases resistance to basal as well as induced oxidative stress by increasing cellular defenses.

DISCUSSION

In this study, we used a cellular model derived from a heterologous epithelial cell line (RK13) in which the expression of murine PrP^C was regulatable in a dose-dependent manner by a doxycycline treatment. Actually most epithelial cell lines we have tested, unlike, RK13, do express PrP.[2] The RK13 cells were chosen because they express no detectable levels of endogenous PrP. The risk that endogenous PrP could interfere with the function of transfected PrP is therefore reduced. This may be a reason why we succeeded in a previous study to infect RK13 cells transfected with ovine PrP (18). It was then logical to generate a clone of cells overexpressing murine PrP, which is used for cell biology and transmission studies. RK13 cells are the only available cell lines allowing a PrP expression from zero to high levels. In the present work we used radioactive copper (^{64}Cu) to study the effect of PrP^C expression on copper binding and uptake.

There is an increasing amount of data supporting a functional role for PrP^C in copper metabolism. First the N-terminal half of PrP^C contains five or six highly conserved octapeptide tandem motifs of the general form PHGGGWGQ, which are capable of binding copper ions with micromolar affinity (7, 8). Indeed, PrP^C isolated from hamster brain can bind a copper affinity column (29). Second, copper content of membrane-enriched brain extract from $PrP^{-/-}$ mice is 10–15-fold lower than in wild type controls while no significant difference was observed for other metals (8). Third, neuronal CuZn-SOD from $PrP^{-/-}$ mice showed decreased activity linked to decreased copper incorporation by the enzyme (10, 30). Neurons cultured from $PrP^{-/-}$ mice were also more sensitive to oxidative stress, perhaps because of the alteration of CuZn-SOD (10). However, the significance of copper binding on PrP^C functions or the role of PrP^C copper metabolism has yet to be clarified. Several hypotheses have been proposed. Copper could have a role in the conformation of the protein (31). PrP^C could have a role in copper transport across the cell membrane, and this could follow different processes. Copper needs to be mobilized from its extracellular ligands (albumin and histidine), and Cu(II) is reduced to Cu(I) by unknown metalloreductases at the membrane surface prior to its delivery across the plasma membrane by the high affinity transporter CTR1 (32). These processes are still unclear. It is also possible that copper may be transported by more than one transport system, at least in some tissues. The cooperative copper-binding mode of PrP^C within the physiological concentration range suggests a role in copper transport (33). So, the contribution of PrP^C, if any, in copper uptake

[2] F. Archer and H. Laude, unpublished data.

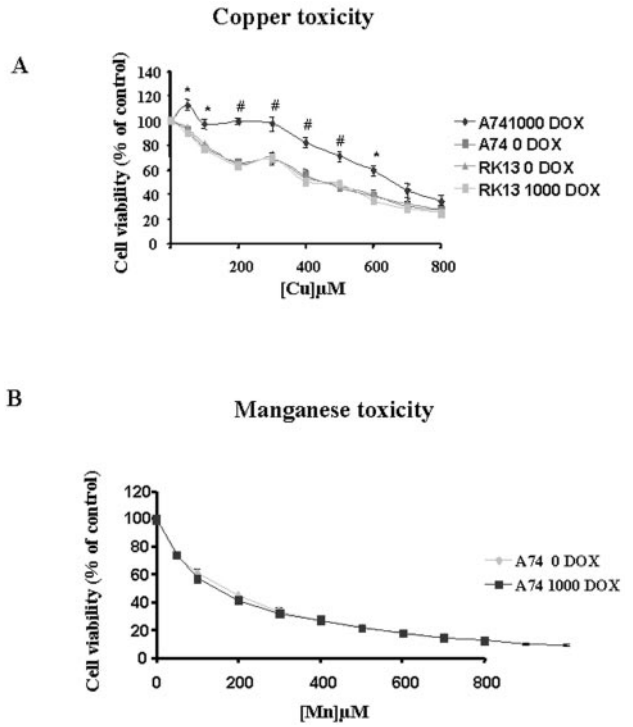

FIG. 6. **PrP^C expression increases resistance to copper but not to manganese toxicity.** Cell lines were incubated with the indicated concentration of copper (*A*) or manganese (*B*) for 24 h, and viability was then measured as described under "Experimental Procedures." Results are expressed as mean percentage ± S.D. of viable cells, assuming 100% viability for untreated A74 cells. *, $p < 0.01$; #, $p < 0.005$.

by cells could be a direct transport across the plasma membrane or a binding step allowing either the mobilization of copper from its ligand or its reduction prior to its effective transport by other systems.

PrP^C is normally attached to the cell membrane via a phosphatidylinositol anchor (34). It has been shown that the enzyme PIPLC releases PrP^C from the cells into media (35). Our *ex vivo* experiments confirm the copper binding activity of the PrP^C protein, because we established a correlation between copper binding and PrP^C expression. This finding was further confirmed when PrP^C was cleaved with PIPLC prior to ^{64}Cu labeling. However, our work does not support that PrP^C could be involved in the copper transport across the membrane, as suggested by studies reporting histidine-dependent uptake of ^{67}Cu proportional to PrP^C expression in cerebellar cells derived from three lines of mice expressing various amounts of PrP^C (36). However, Pauly and Harris (37) have reported that copper stimulates endocytosis of both mouse PrP and chicken PrP on the cell surface of N2a mouse neuroblastoma cells via clathrin-coated pits. They suggested that PrP^C could serve as a recycling receptor for the uptake of copper ions from the extracellular milieu. Also, it has been shown that 100 µM copper resulted in the rapid endocytosis of biotinylated murin PrP^C expressed in human neuroblastoma SH-SY5Y cells (38). In these two studies the minimum concentration of $CuSO_4$ required to produce an observable increase in PrP^C internalization was ~100 µM, which is 15-fold greater than the estimated K_d for binding to synthetic PrP peptides and recombinant PrP (7, 9, 39). In our work we used very low levels of copper, which we believe renders our results much closer to physiological conditions. In any case when high concentrations of copper (100 µM) are used in our cultures the intracellular copper level increased up to 2-fold in both total and soluble fractions in stimulated cells (see Table II). So, only under high copper concentrations PrP^C expression increases copper uptake in the cell. However, our results clearly demonstrated that at physiological concentrations of copper, murine PrP^C binds copper at the outer side of the cell membrane but also indicates that PrP^C does not function as a copper transporter from the extracellular medium to the cytoplasm. This supports the hypothesis that PrP^C may rather be an extracellular copper sensor (33).

Murine PrP^C may serve as a copper chelating or buffering agent in the outer side of the cell membrane, and this may serve to protect cells against toxicity of free copper ions or a copper and reactive oxygen species-dependent cleavage of PrP into the octapeptide repeat region. This process may be related to the function of the molecule in the response to oxidative stress and suggests that the binding of copper is important for its processing (40).

The link between copper and PrP^C may explain the mechanism of neurodegeneration in prion diseases because copper and other transition metals play an important role in the neuropathology of neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), and Amyotrophic lateral sclerosis (ALS) (41). Copper is an important component of various redox enzymes because of its ability to readily adopt two ionic states Cu(I) and Cu(II). Free copper is also a toxic ion, as exemplified by its ability to inactivate proteins through tyrosine nitration, and both deficiency and excess lead to disorders such as Menkes syndrome or Wilson's disease (42), illustrating its physiological importance and duality in the central nervous system. In the absence of copper chelating agent on the cell surface, free copper could react with peroxides such as hydrogen peroxide produced by superoxide dismutation or directly by many enzyme catabolites such as monoamine oxidase, urate oxidase, glucose oxidase, D-amino acid oxidase, and others to form the highly reactive hydroxyl radical (•OH), which can initiate lipid peroxidation as well as protein oxidation and cause apoptosis. It has been shown that in the brain, highest concentrations of PrP^C are found at synapses, and copper binding by PrP^C in the synaptic cleft has a significant influence on synaptic transmission (43). Changes in electrophysiological properties such as long-term potentiation (LTP), circadian rhythm between $PrP^{-/-}$ and wild-type mice could be related to a disturbed copper uptake in $PrP^{-/-}$ mice (43). Stimulated A74 cells undergo high resistance to copper but not to manganese or cadmium toxicity when compared with unstimulated or control cells. This specific protection against copper toxicity may be due to the chelating or buffering effect of murine PrP^C on the cell surface. Previously PC12, cells selected for resistance to copper toxicity and oxidative stress showed high levels of PrP^C (44). Primary cerebellar granule culture derived from PrP knockout mice were significantly more susceptible to H_2O_2 toxicity than wild type; this toxicity was related to a significant decrease in glutathione reductase activity (45) Moreover, increased oxidative damage to proteins and lipids was observed in the brain lysates from $Prnp^{-/-}$ as compared with wild type mice of the same genetic background (46, 47). As oxidative stress has been frequently implicated in neurodegeneration it was very interesting to test the influence of PrP expression in stimulated A74 on antioxidant enzymes activities and resistance to oxidative stress. PrP^C induction in stimulated cells increases significantly CuZn-SOD, catalase, glutathione reductase activities, and glutathione levels in cells. In addition stimulated cells were more resistant to oxidative stress caused by SIN-1. This active metabolite of the vasodilatatory drug molsidomine is frequently used as a model for the continuous release of different free radicals: $O_2^{\bullet-}$, NO•, and/or NO_x, and other potent oxidants such as $ONOO^-$ (48). The

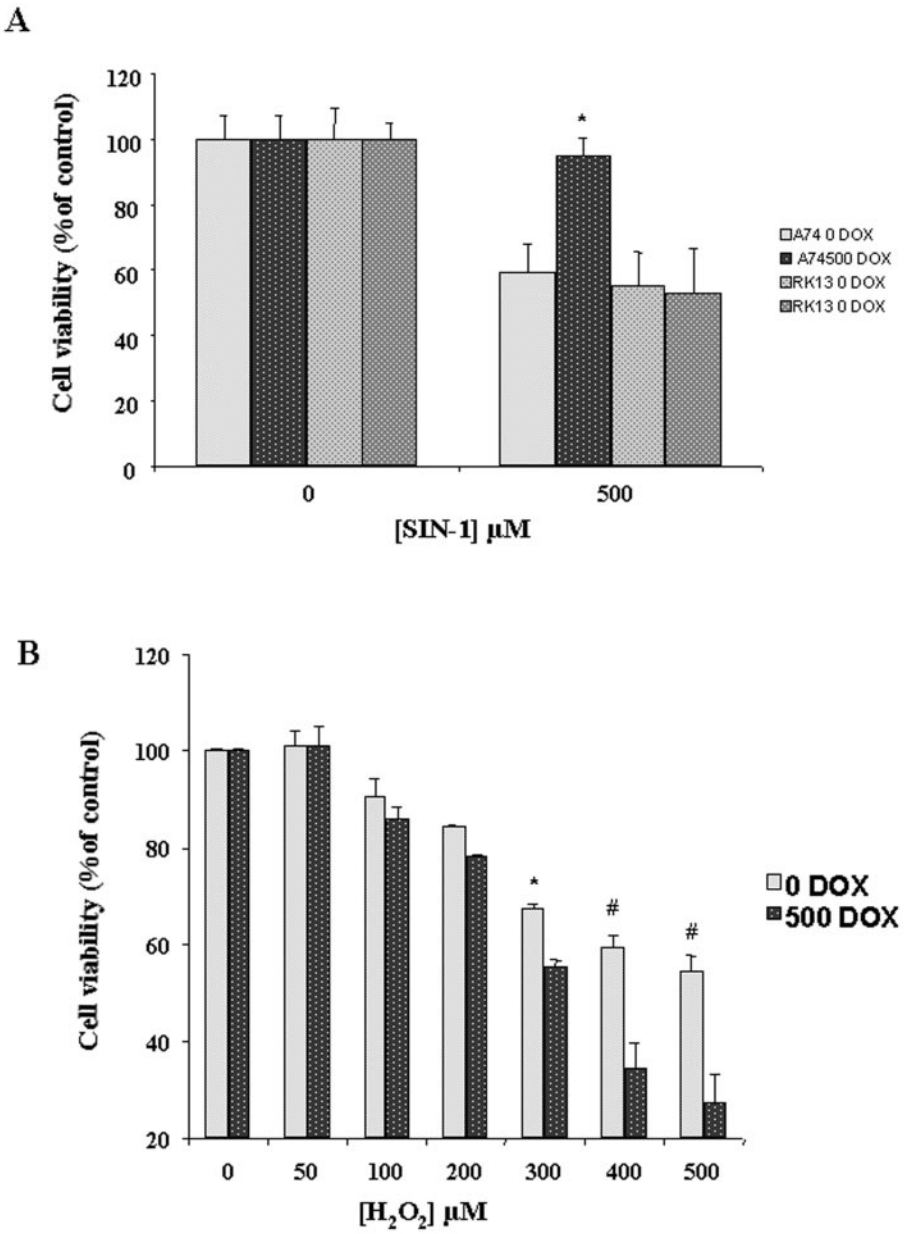

FIG. 7. **PrP^C expression increases resistance to oxidative stress produced by SIN-1 but not to H_2O_2 toxicity.** Cell viability was evaluated by a modified MTT assay as described under "Experimental Procedures" in stimulated and unstimulated A74 and non-transfected RK13 cells after 24 h of exposure to 1 mM SIN-1 (*A*) or after 3 h of exposure to different concentrations of H_2O_2 (*B*). Results are expressed as mean percentage ± S.D. of survival cell; survival was calculated as the percentage of the staining values of untreated cultures. *, $p < 0.01$; #, $p < 0.005$.

TABLE I
PrP^c and antioxidant enzymes activities

Antioxydant enzymes activities were determined in unstimulated, and stimulated A74 (500 ng/ml dox), and untransfected stimulated cells (RK13 500 ng/ml dox) as described under "Experimental Procedures." Data are expressed as the mean ± SD for three independent determinations (*, $p < 0005$; and $, $p < 0.05$). SOD and CAT activities are in unit/mg of protein, GPX and GR activities in unit/g of protein, GSH in µmol/g of protein, and MDA is in nmol/g of protein.

Parameter	A74 0 dox	A74 500 dox	RK13 500 dox
Total SOD	4.5 ± 0.65	5.445 ± 0.55*	4.2 ± 0.67
CuZn-SOD	2.7 ± 0.25	3.445 ± 0.46*	2.42 ± 0.38
Mn-SOD	1.8 ± 0.08	2 ± 0.095	1.78 ± 0.085
Catalase	7.45 ± 0.63	8.75 ± 0.96$	7.29 ± 0.42
GPX	45.6 ± 3.4	49 ± 5.7	43 ± 5.9
GSH	64.59 ± 6.29	114.7 ± 12.16*	70.45 ± 11.8
GR	30.86 ± 2.7	50.58 ± 1.5*	32.2 ± 3.8
MDA	64.3 ± 10.5	40.7 ± 7.4*	59.4 ± 5.8

relationship between PrP^C and oxidative stress arose from results showing an alteration in cellular response to stress with the decrease in PrP^C expression or conversion to the infectious form. PrP$^{-/-}$ mouse brains have reduced CuZn-SOD, and cerebellar cells derived from these mice were more sensitive to oxidative stress (10); increased levels of PrP^C were linked to increased levels of CuZn-SOD activity because of an increase in copper incorporation (12). In our model, we believe that increased CuZn-SOD activity in stimulated cells is due to SOD-like activity of PrP-Cu complexes in the outer side of the cell membrane. Indeed, we detected no change in the protein levels of CuZn-SOD in stimulated and unstimulated cells (Fig. 8), and

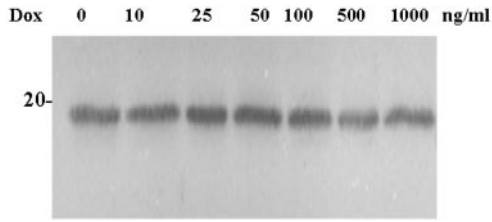

FIG. 8. **PrP^C expression did not change CuZn-SOD protein level.** Cell lysates of stimulated and unstimulated A74 cells were prepared as in the legend to Fig. 1 and the total amounts measured by using the BCA protein assay kit. Equal amounts of proteins were Western blotted with an anti-CuZn-SOD sheep polyclonal antibody. Molecular mass markers are indicated on the *left* in kDa.

TABLE II
Determination of cellular copper content under low and high copper concentrations

Copper concentrations have been determined on either whole cells or lysate supernatants by electrothermal atomic absorption spectrophotometry. Values are expressed as copper concentration normalized to protein content. The standard errors are the standard deviations of the means from several measurements ($n = 3$). (*, $p < 0.05$ *versus* 0 dox; +, $p < 0.05$ *versus* 0 dox + Cu).

Cells	Whole cells	Soluble fraction
0 Dox	8.3 ± 1.4	8.1 ± 2.3
500 Dox	15.5 ± 2.9*	9.4 ± 2.7
0 Dox + 100 μM Cu	467.2 ± 18	410.7 ± 23
500 Dox + 100 μM Cu	1067 ± 38+	863 ± 41+

we demonstrated that copper stays at the outer side of the cell membrane. This antioxidant function in the outer of the cell membrane is very important, especially in neurons, to detoxify free radicals such as O_2^- and other toxic products like peroxynitrite ($ONOO^-$), from reactions between $NO^\cdot$ and O_2^-, which results in dose-dependent neuronal damage. Peroxynitrite has been reported to oxidize protein and nonprotein sulfhydryls (49), to induce membrane lipid peroxidation (50), and nitrotyrosine formation (51). This nitrotyrosine formation markedly inhibits phosphorylation of tyrosine residues *in vitro*, possibly interfering with normal signal transduction pathways (52). Recently PrP^C was involved in signal transduction in neuronal cell cultures (53). In contrast, stimulated cells show decreased resistance to H_2O_2 toxicity. This may be due to both the SOD-like activity of murine PrP (Fig. 7*B*) and to the Fenton reaction via the increased amount of copper bound to the cell membrane. Stimulated cells show a large increase in glutathione levels and glutathione reductase activity. Glutathione, a tripeptide consisting of glycine, cysteine, and glutamic acid moieties, is a major antioxidant and functions directly in the elimination of ROS. Glutathione acts as a cellular redox buffer and even modest variations in GSH concentrations can strongly modulate redox state. It also may be involved in intracellular copper transport and homeostasis by chelating copper ions and diminishing their ability to generate free radicals. This relationship between PrP^C and GSH may be explained by the high resistance to copper toxicity in our cell model when we overexpress murine PrP^C and brain metal perturbations observed in scrapie-infected mice (54).

In conclusion, we have shown that expression of heterologous PrP^C (murine PrP^C) in rabbit kidney cells (RK13) increases copper binding but not uptake, and several antioxidant enzymes activities confer high resistance to oxidative stress. These findings are of major importance since oxidative stress is implicated in several neurodegenerative diseases. We can hypothesize that in prion diseases the conversion of PrP^C to PrP^{Sc} inhibited PrP copper binding. This inhibition could affect, the enzymatic activity of PrP-Cu complexes in the outer of the cell membrane and also, the regulation of the anti-oxidant system, which is PrP-dependent in neurons. In the future, it will be important to determine the influence of PrP conversion on copper binding in a similar model permissive to prion replication (18) and the influence of trace elements such as copper, zinc, or antioxidant on prion diseases as a new therapeutic agent to re-equilibrate the antioxidant deficiencies in these diseases.

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Expression of Prion Protein Increases Cellular Copper Binding and Antioxidant Enzyme Activities but Not Copper Delivery

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