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Cellular prion protein regulates intracellular hydrogen peroxide level and prevents copper-induced apoptosis

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Abstract

The function of cellular prion protein (PrP^C), which is a copper binding protein, remains unclear. To elucidate the mechanisms in which PrP^C is involved in neuroprotection, we compared death signals in prion protein gene-deficient ($Prnp^{-/-}$) primary cerebellar granular neurons (CGNs) to those with wild-type (WT) CGNs. When copper was exposed to these CGNs, ZrchI, and Rikn $Prnp^{-/-}$ CGNs were more sensitized and underwent apoptotic cell death more readily than WT CGNs. Furthermore, the level of intracellular hydrogen peroxide (H_2O_2) in WT CGNs increased by copper toxicity, whereas those in ZrchI and Rikn $Prnp^{-/-}$ CGNs did not. These results suggest that PrP^C modulates the intracellular H_2O_2 level as a copper-binding protein to protect CGNs from apoptotic cell death possibly due to inhibiting a Fenton reaction.

Keywords: Prion protein; PrP-deficient mouse; Copper; Cerebellar granular neurons

The fundamental physiological function of native cellular prion protein (PrP^C) remains unknown. There are several experimental findings related to the prion protein (PrP) function. A considerable amount of data has been accumulated on the response to oxidative stress [1]. In this study, we elucidated the neuroprotective properties of PrP^C in response to copper (Cu) and oxidative stress in the physiological aspect of PrP^C.

PrP^C deficiency results in neuronal phenotypes sensitive to oxidative stress induced by superoxide anion and hydrogen peroxide (H_2O_2) [1–3]. ZrchI PrP gene-deficient ($Prnp^{-/-}$) cerebellar neurons show increased sensitivity to superoxide anion generated by xanthine/xanthine oxidase [2]. Increased sensitivity of ZrchI $Prnp^{-/-}$ neurons to H_2O_2-induced cell death is evident compared to wild-type (WT) neurons [3]. The fact that ZrchI $Prnp^{-/-}$ neurons display lower glutathione reductase (GR) activity, breakdown of H_2O_2 is therefore inhibited [3]. ZrchI $Prnp^{-/-}$ neurons are more sensitive to Cu than WT neurons [4]. Additional neuroprotective effects of PrP have been observed when immortalized neuronal cell lines from Rikn $Prnp^{-/-}$ hippocampal cells are more apoptosis-susceptible to serum withdrawal than WT counterparts [5]. In addition, PrP^C inhibits Bax-induced apoptosis in the primary culture of human neurons [6]. Deletion of the octapeptide repeat domain abolishes this neuroprotective function of PrP^C, and elimination of the glycosyl phosphatidylinositol (GPI)-anchoring sequences elicits no protective effects [6]. Removal of

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serum from neuronal cultures and Bax expression are known to induce intracellular oxidative stress [7,8], and the role of PrP^C in modulating neuronal antioxidant homeostasis has thus been suggested. Prion-infected GT1-7 cells significantly raise the levels of lipid peroxidation, increase sensitivity to glutathione depletion, and attenuate Cu, Zn-superoxide dismutase (SOD), Mn-SOD, GR, and glutathione peroxidase activities [9]. The amounts of lipid and protein oxidation in the brain tissues of *Prnp*$^{-/-}$ mice increase simultaneously, accompanied by decreased SOD activities [10,11]. Despite such significant evidence supporting a neuroprotective role of PrP^C against oxidative stress, the mediatory roles of PrP^C in neuroprotection are still unknown.

Cu binds to PrP at a major Cu(II)-binding site identified as the N-terminal domain; viz., a specific octapeptide region with four sequential repeats [12,13]. PrP^C has a direct role in brain Cu metabolism, with protein-transporting Cu(II) ions from the extracellular milieu acidifying the cellular vacuoles [14]. Contrary to these findings, a recent study has concluded that PrP^C does not participate in the uptake of extracellular Cu(II) at physiological concentrations of Cu [15]. The total brain Cu content in ZrchI *Prnp*$^{-/-}$ mice is not significantly different from that in WT mice [16,17]. In vitro experiments using recombinant mouse and chicken PrP^C refolding to corporate Cu(II) have revealed that PrP^C displays SOD activity [18], which is dependent on the Cu level incorporated into the molecule [18]. Although actual mechanisms of the PrP-related dismutase reaction remain unknown as yet, findings have suggested that neurodegenerative disorders in prion diseases may be caused by abnormalities in Cu metabolism.

Extensive overlaps between systems controlling homeostasis of redox-active metals such as Cu, iron, and oxygen radical metabolisms have been documented [19]. However, the biochemical mechanisms underlying the Cu-induced apoptosis are poorly understood. In this study, we investigated the mechanisms underlying the Cu-induced apoptosis in the primary culture of ZrchI and Rikn *Prnp*$^{-/-}$ cerebellar granular neurons (CGNs). The abnormalities of ZrchI and Rikn *Prnp*$^{-/-}$ CGNs and the mechanisms of neuroprotective effect of PrP^C were analyzed. This study shows that PrP^C regulates the intracellular H_2O_2 level after binding to Cu molecules to prevent neuronal death.

Materials and methods

Animals. ZrchI *Prnp*$^{-/-}$ mice [20] and Rikn *Prnp*$^{-/-}$ mice [21] were used in this study. C57BL/6CrSlc (WT) mice were purchased from Nippon SLC (Hamamastu, Japan).

Reagents. Unless otherwise specified, chemical reagents were obtained from Sigma (St. Louis, MO) and Wako Pure Chemical (Osaka, Japan).

Neuronal cell culture. CGNs were isolated from 6-day-old mice. Following dissociation in Hanks' balanced salt solution and digestion in 0.5% trypsin, cerebella were plated at $1–2 \times 10^6$ cells/cm^2 in poly-L-lysine (PLL) coated dishes (Falcon). Cultures were maintained in Neurobasal media (NB; Gibco) supplemented with B27, 25 mM KCl, 2 mM glutamine, and 1% antibiotics (penicillin, streptomycin). Cultures were maintained at 37 °C in atmosphere with 5% CO_2.

Cell viability was determined on the last day of the experiments. In brief, 3-(4,5-dimetyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) was diluted to 200 μM and added to cultures for 2 h at 37 °C. The MTT-formazan product was released from cells by adding isopropanol before being measured by 570-nm spectrophotometry. Survival ratios compared to non-treated controls were determined.

Detection of apoptosis. DNA fragmentation was detected by the DNA ladder assay [22]. Harvested cells were prepared with lysis using lysis buffer [10 mM Tris–HCl (pH 7.4), 10 mM EDTA (pH8.0), and 0.5% Triton X-100] for 20 min on ice before centrifugation (12,000*g*, 30 min). The aqueous phase was incubated with 400 μg/ml DNase-free RNase A (Nippongene, Tokyo Japan) for 1 h at 37 °C. Proteins were digested with 400 μg/ml proteinase K for 1 h at 37 °C before DNA was precipitated overnight in aqueous 0.4 M NaCl containing 50% isopropanol at −20 °C. Precipitated DNA samples were resuspended in TE buffer and electrophoresed in 2% agarose gel. The gel was stained with ethidium bromide (0.5 μg/ml) for 10 min and destained with ultra-purified water for 10 min. DNA bands visualized by a UV light transilluminator were photographed (Bio-Rad, Cambridge, MA) accordingly.

Nuclear morphological analysis. Apoptosis was assessed by staining cell nuclei with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; Dojindo, Kumamoto, Japan) based on methods adapted from those previously described [23]. Cells with degenerating nuclei were thus discriminated. Briefly, cells were fixed for 20 min in fresh 4% paraformaldehyde in PBS(−), rinsed with PBS(−), before staining for 15 min with 3 μM DAPI in PBS(−). After being washed twice, cells were scored for chromatin condensation by fluorescence microscopy using a fluorescein filter (330–380 nm excitation). Total and apoptotic nuclei were counted. In all cases, >100 cells were counted per well. At least three different cell cultures utilizing four separate wells were employed.

PrP^C expression by flowcytometry. To examine PrP^C expression, flowcytometry was used as previously described [22]. Briefly, collected cells were initially incubated with 6H4 antibody (Prionics, Zurich, Switzerland) as the primary antibody and detected with FITC-labeled secondary antibody before analysis with a flowcytometer (FACScan).

Measurement of intracellular H_2O_2. Intracellular H_2O_2 levels were determined based on methods adapted from those previously described [24,25]. Cells were seeded in 6-cm culture dishes at 1×10^5 cells/well. Cells were incubated in various conditions. After the incubation, the cells were loaded with a final concentration of 10 μM of 2′7′-dichlorofluorescein diacetate (DCFH-DA) (Lambda Fluoreszenztechnologie, Graz, Austria) delivered in serum-free media for 15 min at 37 °C. DCFH-DA was incorporated into viable cells and hydrolyzed by intracellular elastase, generating the non-fluorescent molecule DCFH. Intercellular H_2O_2 oxidized DCFH to yield the fluorescent DCF molecule. Cells collected by pipetting without washing were subsequently analyzed. The fluorescence of each well was measured by a flowcytometer (480 nm excitation and 530 nm emission wavelengths). The total number of events was >5000 per sample.

Statistical analysis. Individual cultures were performed in triplicate. The results expressed as the means ± SEM for the number of culture preparations are indicated in the respective figures. Statistical analysis of results was performed by the non-paired Student's *t*-test. In cases where $p < 0.05$, the differences were considered significant.

Results and discussion

Previous study has shown that ZrchI *Prnp*$^{-/-}$ cerebellar cells are more sensitive to Cu toxicity than WT

cerebellar cells [4]. To further characterize the cell death in *Prnp*$^{-/-}$ cells exposed to Cu, the differences of DNA fragmentation in apoptosis between Rikn *Prnp*$^{-/-}$ and WT CGNs were examined. Rikn *Prnp*$^{-/-}$ and WT CGNs were cultured in $CuCl_2$ containing media (NB/B27) for 48 h, and samples were analyzed by DNA ladder assay. Exposure of Cu to CGNs showed significantly more cell deaths in *Prnp*$^{-/-}$ neurons compared with WT CGNs. The exposure of cultivated cells to 100–300 μM Cu resulted in more apoptotic cell death in Rikn *Prnp*$^{-/-}$ CGNs than WT CGNs (Fig. 1A). The CGN viability was assayed by the normal and apoptotic (condensed and fragmented) DAPI-stained nuclei were counted under fluorescence microscopy. More than 70% of Rikn *Prnp*$^{-/-}$ CGNs and less than 40% of WT CGNs incubated for 24 h in Cu (300 μM)-containing media showed bright pyknotic soma with disintegrated neurite under phase-contrast microscopy (data not shown), and ca. 80% of Rikn *Prnp*$^{-/-}$ CGNs and ca. 50% of WT CGNs exhibited condensed or fragmented nuclei when stained with DAPI, manifesting typical features of apoptosis (Fig. 1B).

Of Cu toxicities observed in WT, ZrchI *Prnp*$^{-/-}$, and Rikn *Prnp*$^{-/-}$ CGNs in NB/B27, the ZrchI and Rikn *Prnp*$^{-/-}$ CGNs were more sensitive than WT CGNs (Fig. 2). Cu-containing media were significantly more toxic to ZrchI and Rikn *Prnp*$^{-/-}$ CGNs than WT CGNs after 48-h exposure at 150 and 200 μM (Fig. 2). Next, as Cu is a redox active metal in a Fenton reaction, which produces hydroxyl radicals from H_2O_2, intracellular H_2O_2 was monitored to ascertain if Cu was involved in a Fenton

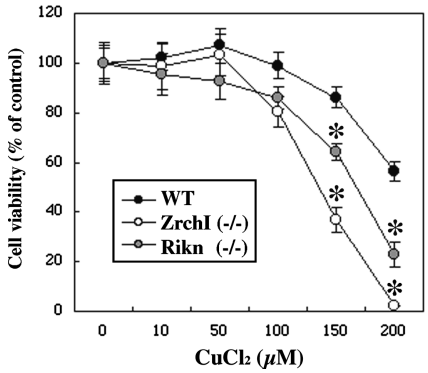

Fig. 2. Effect of Cu on cell viability in primary cultures of wild-type (WT), ZrchI and Rikn *Prnp*$^{-/-}$ cerebellar granular neurons (CGNs). Seven-day-old primary CGNs of WT, ZrchI, and Rikn *Prnp*$^{-/-}$ mice were exposed to $CuCl_2$ for 48 h, respectively, and cell viabilities were then determined using the MTT assay. ZrchI and Rikn *Prnp*$^{-/-}$ CGNs in NB/B27 medium were significantly more susceptible to 150 and 200 μM $CuCl_2$ toxicity than WT CGNs after 48-h exposure. Differences where $p < 0.05$ (*) were significant when compared with WT CGNs.

reaction-mediated cell damage in WT, ZrchI or Rikn *Prnp*$^{-/-}$ CGNs. By means of fluorochrome DCFH-DA coupled with flowcytometric technique, the level of intracellular H_2O_2 was monitored. Cu-treated WT CGNs increased H_2O_2 in a time-dependent manner with the peak established at 5 h after Cu-exposure (Fig. 3), whereas, at any tested time or concentration, signals indicating H_2O_2 increases were not detected in ZrchI and Rikn *Prnp*$^{-/-}$ CGNs. This finding indicated that the H_2O_2 production of WT CGNs was more highly induced by Cu

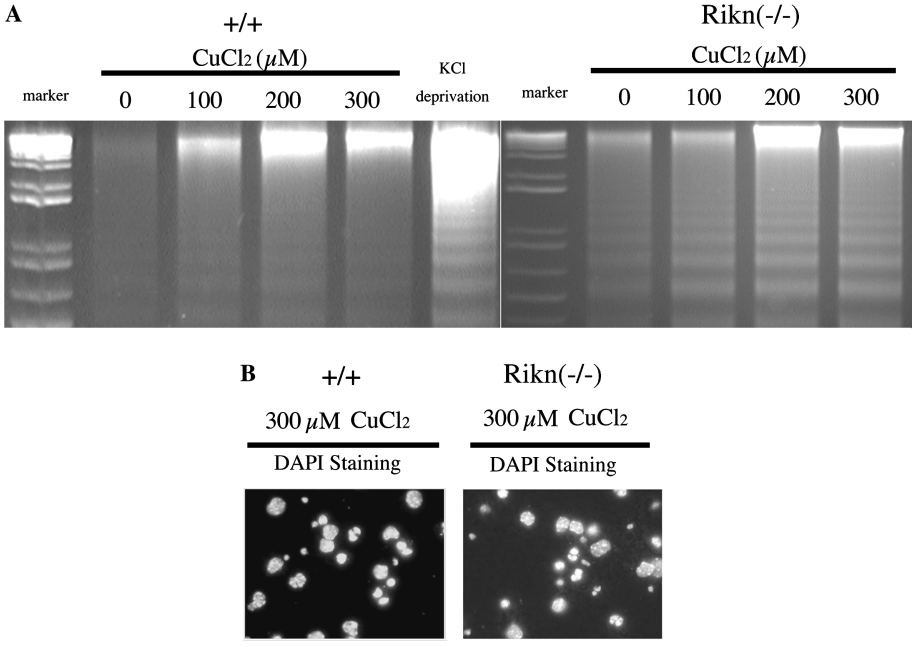

Fig. 1. Detection of Cu-induced apoptosis in WT and *Prnp*$^{-/-}$ cerebral granular neurons (CGNs). (A) CGNs derived from WT and Rikn *Prnp*$^{-/-}$ mice were cultured in media with the indicated concentrations of $CuCl_2$ or 5 mM KCl for 48 h and analyzed by the DNA fragmentation assay. (B) Alteration in nuclear morphology was visualized by DAPI staining after incubation of WT and Rikn *Prnp*$^{-/-}$ CGNs with 300 μM $CuCl_2$ for 24 h.

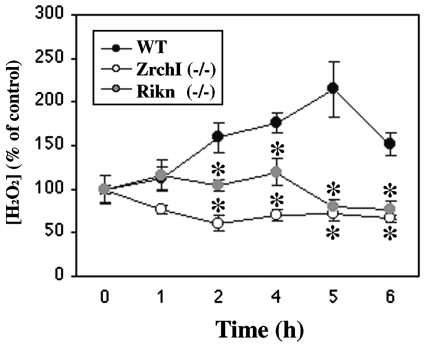

Fig. 3. Evaluation of intracellular H_2O_2 production in WT, ZrchI, and Rikn *Prnp*$^{-/-}$ cerebellar granular neurons (CGNs) after treatments with Cu. WT, ZrchI, and Rikn *Prnp*$^{-/-}$ CGNs were cultured in media containing 300 μM $CuCl_2$ for the indicated time. DCFH-DA was added to the culture medium 30 min after incubation. CGNs collected by pipetting were analyzed by flowcytometry. Intracellular H_2O_2 levels of WT CGNs were increased by Cu, but not those of ZrchI and Rikn *Prnp*$^{-/-}$ CGNs. Differences where $p < 0.05$ (*) were significant when compared with WT CGNs.

than those of ZrchI and Rikn *Prnp*$^{-/-}$ CGNs, suggesting that increase of intracellular H_2O_2 induced by Cu toxicity requires PrP^C expression, and intracellular H_2O_2 in *Prnp*$^{-/-}$ CGNs was effectively converted into hydroxyl radicals by a Fenton reaction. Finally, we investigated the effect of Cu on PrP^C expression in WT CGNs. Flowcytometry revealed that PrP^C expression significantly decreased from cell surface by 6-h exposure of Cu in a dose-dependent manner (Fig. 4).

Accumulating evidence suggests that PrP^C may serve as an antioxidant and/or a Cu-transporting agent. PrP^C has been shown to have an antioxidative and/or anti-apoptotic effect in *Prnp*$^{-/-}$ cell lines [23] and ZrchI *Prnp*$^{-/-}$ neurons [2]. Therefore, we conducted experiments using Rikn *Prnp*$^{-/-}$ mice, which ectopically express PrP-like protein PrPLP/Dpl, [17] to examine whether Rikn *Prnp*$^{-/-}$ neurons are also sensitive to

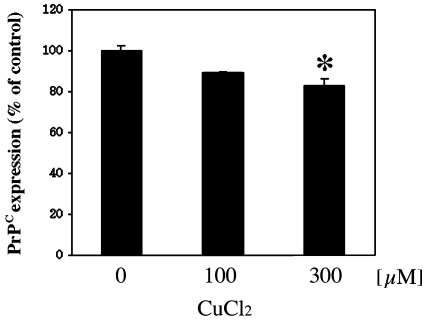

Fig. 4. Decreased expression of PrP^C induced by Cu treatment in WT cerebellar granular neurons (CGNs). PrP^C expression of WT CGNs treated for 6 h with the indicated concentrations of $CuCl_2$ was analyzed by flowcytometry with anti-PrP antibody as described in Materials and methods. Values are expressed as means ± SEM ($N = 3$). Differences where $p < 0.05$ (*) were significant when compared with untreated WT CGNs.

oxidative or apoptotic stress. Rikn *Prnp*$^{-/-}$ CGNs were more susceptible to Cu-induced apoptosis than WT CGNs. There was no difference in viability between WT and Rikn *Prnp*$^{-/-}$ CGNs exposed to any concentration (0, 10, 50, 100, 150, 200, or 300 μM for 48 h) of $ZnCl_2$, $NiCl_2$, or $FeCl_2$ tested (data not shown). Therefore, different from other metals, Cu toxicity on Rikn *Prnp*$^{-/-}$ cells displays a unique perspective.

In this study, Cu-induced oxidative DNA damage and apoptosis in *Prnp*$^{-/-}$ CGNs. The pathways of death signals were investigated to clarify the neuroprotective roles of PrP^C against Cu-promoted apoptosis. Cu induces hydroxyl radicals generated by a Fenton reaction from H_2O_2, resulting in apoptosis [26]. As increase of intracellular H_2O_2 requires PrP^C expression, PrP^C may suppress apoptosis by inhibiting a Fenton reaction and formation of hydroxyl radicals. Decrease of PrP^C expression at cell membrane by Cu is in accordance with a previous report that Cu induces endocytosis of PrP^C [14]. Intracellular PrP^C may have an effect on intracellular H_2O_2 level. Recent studies have reported that Cu toxicity is enhanced by certain oxidants that affect the redox state of Cu. For example, ascorbic acid reduces Cu^{2+} to Cu^+ ion to facilitate ion uptake, thus leading to a loss in cell viability [27]. In cells, L-DOPA, dopamine, and 3-*O*-methyl DOPA inflict extensive oxidative DNA damage in the presence of H_2O_2 and traces of Cu ions [28]. PrP^C-bound Cu is related to the formation of carbonyls by dopamine [29]. Therefore, it is most likely that PrP^C mediates activity of these oxidants via a concurrent dual-action; viz., direct inhibition of the increase in a Fenton reaction coupled with indirect attenuation of the copper toxicity.

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