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PrP cooperates with STI1 to regulate SOD activity in PrP-deficient neuronal cell line

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Abstract

Cellular prion protein (PrP^C) plays anti-apoptotic and anti-oxidative roles in apoptosis induced by serum deprivation in an immortalized prion protein gene (*Prnp*)-deficient neuronal cell line. The octapeptide repeat region (OR) and N-terminal half of the hydrophobic region (HR) of PrP^C are indispensable for PrP^C activity, but the mechanisms remain unclear. In the present study, elucidation of the mechanisms by which PrP^C elicits the anti-oxidative activities was facilitated by evidence of stress-inducible protein 1 (STI1) mediating PrP^C-dependent superoxide dismutase (SOD) activation. Immunoprecipitation revealed that PrP^C was associated with STI1. The inhibitory peptides against PrP^C–STI1 binding [STI1 pep.1 and PrP(113–132)] indicated toxic activity in PrP^C-expressing cells by inhibiting SOD activity but not in $Prnp^{-/-}$ cells. Furthermore, OR and N-terminal half of the HR were required for the inhibitory effect of PrP(113–132) but not STI1 pep.1. These data are consistent with results established with a model where OR and N-terminal half of the HR mediate the action of STI1 upon cell survival and upregulation of SOD activity.

Keywords: Prion disease; Prion protein; Apoptosis; PrP-deficient cell line; STI1

Transmissible spongiform encephalopathies (TSE) are fatal neurological disorders that include the Creutzfeldt–Jakob disease and Gerstmann–Sträussler Scheinker syndrome in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle [1]. The pathological characteristics of the diseases encompass neuronal cell loss, vacuolation, astrocytosis, and amyloid plaques in the brain [1]. After prion infection, cellular prion protein (PrP^C) is converted into an abnormal isoform of prion protein (PrP^{Sc}), which has been proposed to be responsible for the disease [1]. Therefore, PrP^C is hypothetically required to induce the disease and eventually provoke neural damages. The fact that prion protein (PrP) gene (*Prnp*)-knockout mice are resistant to infectivity and toxicity induced by pathogenic inoculates [2] is clear evidence of such a hypothesis.

Several reports are devoted to the putative physiological function of PrP^C. Aberrant circadian rhythms [3], electrophysiological abnormalities [4], and high susceptibility to seizure [5] in $Prnp^{-/-}$ mice have been reported. Recently, participation of PrP^C in the inhibition of apoptosis has been demonstrated. Although removal of serum from cell cultures causes apoptosis in *Prnp*-deficient immortalized hippocampal neuronal cells, transfection

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of *Prnp* suppresses the apoptosis of $Prnp^{-/-}$ cells under serum-free conditions [6]. We have further demonstrated that reintroduction of *Prnp* upregulates superoxide dismutase (SOD) activity and inhibits superoxide generation, suggesting that PrP^C suppresses apoptosis by upregulation of SOD activity [7]. However, PrP^C lacking an octapeptide repeat region (OR) or an N-terminal half of the hydrophobic region (HR) loses its anti-apoptotic and anti-oxidative functions [7,8]. Several potential mediators of PrP^C signals have been recently reported. Copper specifically binds the OR of PrP^C [9] and enhances endocytosis of PrP^C [10]. A PrP^C-binding molecule, stress-inducible protein 1 (STI1), binds with amino acid residues 113–128 located in the N-terminal half of HR of PrP^C [11]. These studies prompted us to perform additional studies in order to determine how the STI1 might contribute to PrP^C-dependent anti-oxidative signaling.

To investigate whether the STI1 is important for the biological activities displayed by PrP^C, the effect of the inhibitory peptides against PrP^C–STI1 binding on $Prnp^{-/-}$ cells was compared to that on PrP^C-expressing $Prnp^{-/-}$ cells under serum-free conditions. The inhibitory peptides are toxic to PrP^C-expressing cells by inhibiting the SOD activity, although such is not the case for $Prnp^{-/-}$ cells. Furthermore, immunoprecipitation indicated that STI1 interacted with PrP^C in PrP^C-expressing cells. Therefore, we propose that STI1 involves in PrP^C-dependent SOD activation that can inhibit apoptosis.

Materials and methods

Cell cultures and animals. Murine *Prnp*-deficient neuronal cells HpL3-4 [6] and transfectants including HpL3-4-EM [8], HpL3-4-PrP [8], HpL3-4-Δ#1 [8], HpL3-4-Δ#2 [8], and HpL3-4-Δ#3 [8] cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 incubator. Unless otherwise specified, serum deprivation was performed as previously described [7]. Occasionally, peptides [PrP(113–132) peptide: GAAAAGAVVGGLGGYMLGSA corresponding to p10 peptide [11] and residues 113–132 of mouse PrP; STI1 pep.1: ELGNDAYKKKDFDKAL corresponding to residues 230–245 of mouse STI1 [11]] synthesized and purified by American Peptide Company (Sunnyvale, CA) were added to the media for inhibition of PrP^C–STI1 binding.

Preparation of cellular fractions. To fractionate cell samples, the cell homogenate was prepared in phosphate-buffered saline (PBS) by sonication. After centrifugation at 600*g* for 15 min at 4 °C, supernatants were further ultracentrifuged at 200,000*g* for 1 h at 4 °C. The pellets and supernatants were solubilized in a radio-immunoprecipitation assay (RIPA) buffer to yield membrane and soluble fractions. The RIPA buffer was composed of 10 mM Tris–HCl (pH 7.4) containing 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.15 M sodium chloride supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF).

SOD activity assay. Cells were sonicated in ice-cold RIPA buffer supplemented with PMSF and centrifuged at 15,000*g* for 5 min at 4 °C. Protein concentrations of the supernatants were measured by the DC protein assay (Bio-Rad, Hercules, CA). Each protein extract (20 μg) was assayed by the SOD assay kit-WST (Dojindo, Kumamoto, Japan). The SOD activity was compared with 1 U of bovine erythrocyte Cu/Zn-SOD (Sigma S2515) activity and estimated using the standard curve of SOD activity versus absorbance at 450 nm. The SOD activity was expressed as U/mg protein.

Immunoprecipitation. HpL3-4-EM and HpL3-4-PrP cells were resuspended in RIPA buffer, sonicated on ice, and centrifuged at 15,000*g* for 5 min at 4 °C. The supernatants were precleared using protein G–Sepharose beads suspension (Amersham–Pharmacia Biotech, Piscataway, NJ) for 1 h at 4 °C, and then incubated with 2.5 μg SAF32 for 1 h at 4 °C. Next, 20 μl of protein G–Sepharose beads suspension was added to each of the immunocomplexes and the mixtures were rotated for 1 h at 4 °C. After washing four times with the RIPA buffer, the immunoprecipitated proteins were subjected to SDS–PAGE and Western blotting.

Western blot assay. The Western blot assay was performed as described previously [12]. Briefly, cell lysates were prepared in RIPA buffer. The protein concentration was measured using the Bio-Rad DC assay, and SDS/polyacrylamide gel electrophoresis was conducted before electrical transfer onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham–Pharmacia Biotech). PrP, STI1 or Cu/Zn-SOD was detected as described previously [12] with anti-PrP 6H4 (Prionics, Zürich, Switzerland) [13], anti-PrP SAF83 (SPI Bio, Montigny le Bretonneux, France), anti-recombinant mSTI1 [11] or anti-Cu/Zn-SOD (Stressgen, Victoria, BC) antibody and horseradish peroxidase-conjugated secondary antibody. The probed proteins were detected using an enhanced chemiluminescence detection kit (Amersham–Pharmacia Biotech).

Cell survival assay. In cell survival assays, cells were seeded on 96-well plates at 5000 cells/well. Two days later, cells were washed twice with serum-free DMEM followed by incubation in serum-free DMEM or 10% FCS–DMEM. Incubation of PrP(113–132) or STI1 pep.1 peptides was carried out in serum-free DMEM plus peptides. Viable cell counts were estimated by the Tetra Color One cell proliferation assay system (Seikagaku Kogyo, Tokyo, Japan). In this assay system, the sodium salt of 4-[3-(iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate, which is a water-soluble tetrazolium (WST), was utilized to count the viable cells. It has been reported that the cell viability assay using WST produces formazan dye that correlates linearly with the number of viable cells over the range of 1000–50,000 cells/well [14]. As such, cells were treated with WST (10 μl/well) before further incubation for 4 h. Absorbance at 450 nm was measured to quantify the level of formazan by a microplate reader (Bio-Rad).

Results and discussion

To examine the mechanism by which PrP^C prevents apoptosis and upregulates SOD activity, we investigated whether STI1 (reported to bind PrP^C in the HR [11]) could mediate PrP-dependent SOD activation. We first examined the localization of PrP^C, STI1, and Cu/Zn-SOD in HpL3-4 cells in the absence or presence of PrP^C, followed by analysis of the distributions of PrP^C, STI1, and Cu/Zn-SOD by cellular fractionation. The resulting precipitate after ultracentrifugation (200,000*g* for 1 h at 4 °C) of the cell homogenate (considered to be the membrane fraction) and the supernatant (the soluble fraction) were subjected to Western blot analysis with anti-PrP 6H4, anti-recombinant STI1, and anti-Cu/Zn-SOD antibody (Fig. 1). PrP^C was detected only in the membrane fraction of HpL3-4-PrP cells, whereas Cu/

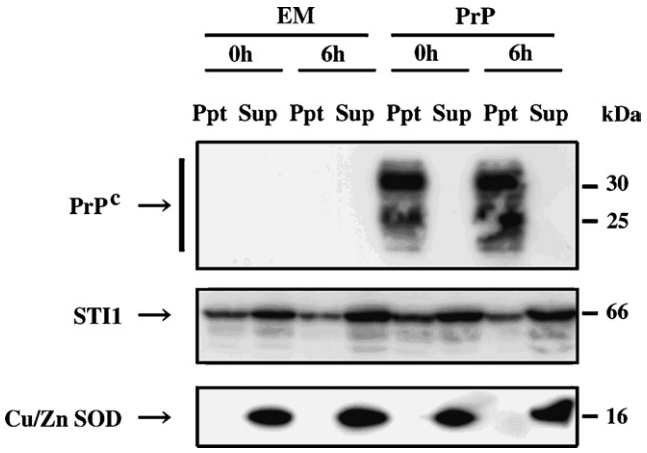


Fig. 1. Localization of STI1 is not altered by PrP expression. STI1 (66 kDa) was detected in fractionated membrane fraction (Ppt) and soluble fraction (Sup) in HpL3-4-EM and HpL3-4-PrP cells previously subjected to serum deprivation for 0 or 6 h (see Materials and methods). The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and detected with anti-PrP 6H4, anti-mSTI1 or anti-Cu/Zn SOD antibody.

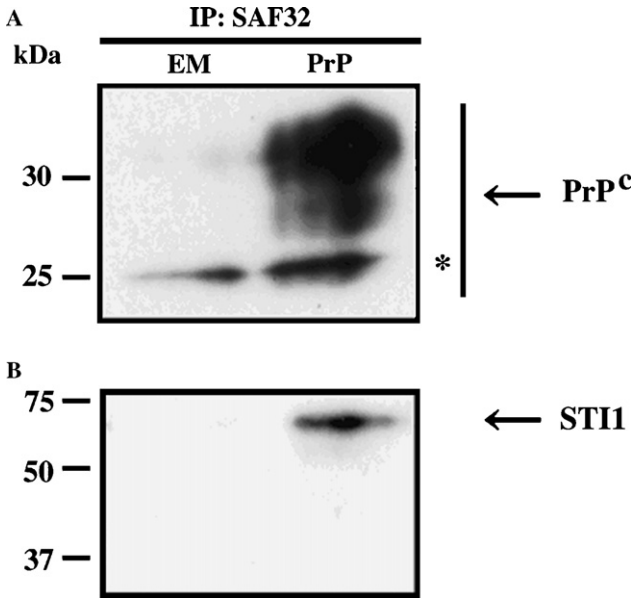


Fig. 2. PrP^C interacts with STI1. HpL3-4-EM and HpL3-4-PrP cells were immunoprecipitated with anti-PrP SAF32. The immunoprecipitates were then immunoblotted with anti-PrP SAF83 (A) or anti-recombinant STI1 (B) antibody. The asterisk represents the immunoglobulin light chain.

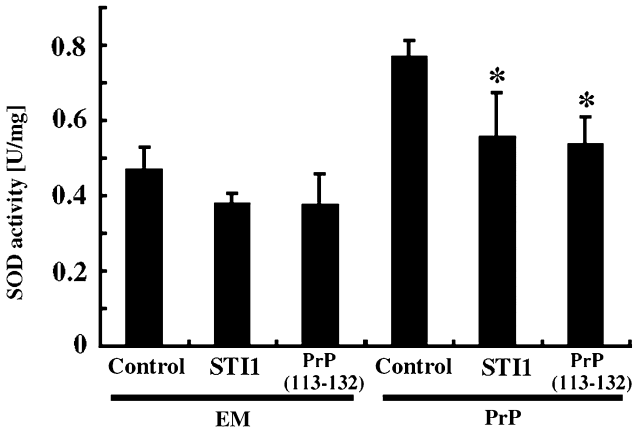
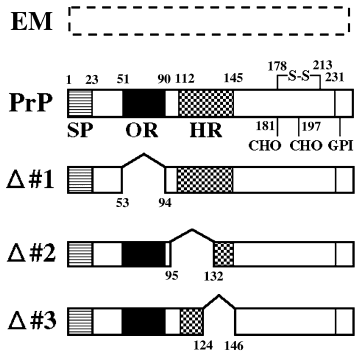


Fig. 3. Inhibitory effect of STI1 and PrP^C-binding peptide on SOD activity. The SOD activities of HpL3-4-EM and HpL3-4-PrP cells previously serum-deprived for 6 h in the absence (Control) or presence of STI1 pep.1 (5 μM) or PrP(113–132) peptide (4 μM) were measured as described in Materials and methods. Values are expressed as means ± SEM ($N = 4$). Differences where $p < 0.01$ (*) versus Control were considered significant when verified by the non-repeated measures ANOVA followed by the Bonferroni correction.

Zn-SOD was detected in the soluble fraction of HpL3-4-EM and HpL3-4-PrP cells. STI1 was detected in both the membrane and soluble fractions in HpL3-4-EM and HpL3-4-PrP cells in the absence and presence of serum. The expression level and ratio of STI1 protein in the soluble fraction were compared with those in the membrane fraction in HpL3-4-EM and HpL3-4-PrP cells. STI1 was detected as doublet or triplet with lower molecular weight bands, which mobilized from the membrane to the soluble fraction upon serum deprivation in HpL3-4-EM and HpL3-4-PrP cells, suggesting the dephosphorylated form of STI1 protein [15–17]. We next examined if PrP^C would bind to STI1 in HpL3-4-PrP cells. Interestingly, coimmunoprecipitation with SAF32 (Fig. 2) indicated that PrP^C bound to STI1 in HpL3-4-PrP cells but not HpL3-4-EM cells.

The PrP(113–132) peptide (corresponding to p10 peptide [11] that contains the STI1-binding domain, i.e., amino acid residues 113–128 of mouse PrP [11]) and STI1 pep.1 peptide (corresponding to amino acid residues 230–245 of STI1 [11] or the PrP^C-binding sites) were used for the third analysis. The PrP(113–132) and STI1 pep.1 peptides have already been reported to inhibit PrP^C–STI1 interaction, and STI1 pep.1 reduces anisomycin-induced cell death in retinal explants from neonatal mice and rats [11]. STI1 pep.1 and PrP(113–132) peptides significantly inhibited SOD activity of HpL3-4-PrP cells but not HpL3-4-EM cells (Fig. 3). Furthermore, STI1 pep.1 and PrP(113–132) peptides inhibited the cell survival in HpL3-4-PrP cells without influencing the cell survival of HpL3-4-EM cells (Fig. 4). The effect of peptides on cell survival of HpL3-4 cells expressing deletion mutants was also investigated (Fig. 4). STI1 pep.1 peptide inhibited cell survival of HpL3-4 cells expressing PrP with removal of the OR (HpL3-4-Δ#1), the N-terminal half of HR (HpL3-4-Δ#2) or the C-terminal half of HR (HpL3-4-Δ#3). In contrast, PrP(113–132) peptide inhibited cell survival of HpL3-4-Δ#3 cells, while the peptide did not inhibit those of HpL3-4-Δ#1 and HpL3-4-Δ#2 cells.

Although numerous attempts to elucidate the prion-related pathogenic mechanism(s), limited data concerning the putative physiological function of PrP^C have



	Inhibition [%]	
	STI1	PrP(113-132)
EM	4.6	0
PrP	100	100
Δ#1	84.1	26.5
Δ#2	69.9	0
Δ#3	141.4	167.5

Fig. 4. Inhibitory effect of STI1 and PrP^C-binding peptide on cell death. Schematic presentations of PrP deletion mutants of mouse PrP [PrP: wild-type PrP; Δ#1: PrP(Δ53–94, Q52H); Δ#2: PrP(Δ95–132); and Δ#3: PrP(Δ124–146)] are shown on the left. Schematic locations of the deletions as compared with the wild-type protein are shown by a space within the bar next to the indicated protein. Numbers refer to the amino acid residues in the mouse PrP sequence. The disulfides (S–S), two Asn-linked glycosylation sites (CHO), signal peptide sequence (SP), octapeptide repeat region (OR), hydrophobic region (HR), and glycosylphosphatidylinositol anchor (GPI) are shown. HpL3-4 cells expressing wild-type PrP (PrP: HpL3-4-PrP), PrP(Δ53–94, Q52H) (Δ#1: HpL3-4-Δ#1), PrP(Δ95–132) (Δ#2: HpL3-4-Δ#2), PrP(Δ124–146) (Δ#3: HpL3-4-Δ#3) or the empty vector per se (EM: HpL3-4-EM) cells were serum-deprived at 6.25 μM STI1 pep.1 or PrP(113–132) peptides. The inhibitory effect of the peptides on cell survival was examined 48 h later by the Tetra Color One cell proliferation assay. The rate of inhibition of HpL3-4-PrP cells by STI1 pep.1 or PrP(113–132) peptide was taken as 100%, respectively.

been documented. In a previous analysis of PrP, we have reported that serum deprivation of cell cultures induces apoptosis in a *Prnp*-deficient immortalized hippocampal cell line but not in *Prnp*-transfected $Prnp^{-/-}$ cells [6]. Furthermore, deletion analysis of PrP^C has demonstrated that deletion of the OR or N-terminal half of the HR induces apoptosis under serum-free conditions [7,8]. As the OR and HR of PrP are well conserved among mammals [18,19], the high evolutionary conservation of these regions is consistent with the notion that these regions are the important domains for PrP functions.

In this study, we investigated the possibility of PrP^C regulating the cellular SOD activity by interaction with STI1, which binds HR of PrP [11]. In addition, PrP has recently been reported to mediate neuroprotective signals [11]. While Lassle et al. [15] have localized murine STI1 in cytoplasm of NIH3T3 cells, Zanata et al. [11] have located STI1, at least in part, in the cell membrane of HEK293T cells. In the present study, STI1 was detected in the soluble and membrane fractions of HpL3-4-EM and HpL3-4-PrP cells. Furthermore, STI1 pep.1 and PrP(113–132) peptide, which inhibit PrP^C–STI1 binding [11], prevented the SOD activity and promoted cell death in HpL3-4-PrP but not in HpL3-4-EM cells. Moreover, immunoprecipitation demonstrated that PrP^C was associated with STI1 in HpL3-4-PrP cells. These results suggest two possibilities: (i) direct interaction between PrP and STI1 involves PrP-dependent SOD activation, leading to the inhibition of cell death, and (ii) copper may transduce PrP-dependent SOD activation mediated by STI1. In (i), as a transmembrane domain or a signal-peptide domain was not found in STI1, the mechanism(s) of STI1 interaction with PrP^C remains unclear. Both STI1 and PrP^C could be phosphorylated, and phosphorylation may therefore be related to the signal transduction [15,20]. As PrR peptide, which has a high resemblance to the hydropathic profile of the STI1-binding site for PrP, transduces the cAMP/protein kinase A (PKA) pathway [21], STI1 probably mediates the cAMP/PKA pathway by phosphorylation. As for (ii), it is supported by the important roles played by copper in PrP^C and Cu/Zn-SOD activity [22,23]. Close coordination with the latter recent data from our laboratories has suggested that PrP^C stabilizes cellular copper concentration under oxidative conditions [24]. Furthermore, as not only STI1-binding site but also OR is needed for STI1-mediated anti-apoptotic signal, suggesting that copper binding to PrP plays essential role for the signal. However, the binding of copper to recombinant PrP does not influence its ability to interact with STI1 [11]. Therefore, further studies on the role(s) of STI1–PrP^C interaction in relation with copper are warranted to fully elucidate the anti-apoptotic functions of PrP^C. Interestingly, PrP(113–132) peptide did not inhibit the cell survival of HpL3-4-Δ#1 and HpL3-4-Δ#2 cells. These results suggest that not only N-terminal half of HR, which includes STI1-binding site, but also OR are required for STI1-mediated anti-apoptotic signals. Moreover, as STI1 pep.1 but not PrP(113–132) peptide inhibited cell survival of HpL3-4 cells expressing all of the PrP deletion mutants, PrP^C suggests to mediate the action of STI1 upon cell survival but not vice versa. Finally, it should be noted that the concentration of pep.1 and PrP (113-132) peptide needed in interaction as an interfering peptide to induce a neuroprotective effect on nervous tissues is lower than those required to induce pro-degenerative effects in our in vitro study [11]. According to the results, it is possible that the reaction against the interfering peptides may depend on the type of cells or neurons.

In summary, PrP^C indicated an anti-apoptotic function in cells [6–8]. The anti-apoptotic function of PrP^C is mediated by upregulation of cellular SOD, while STI1 is involved in the PrP^C-dependent SOD activation. This activation is abrogated by STI1 pep.1 and PrP(113–132) peptide inhibiting the PrP^C–STI1 binding, as a result of direct interaction between PrP^C and STI1 (because PrP^C associates with STI1 in this cell system). Furthermore, the OR and N-terminal half of HR, which harbor the STI1-binding site, are indispensable for

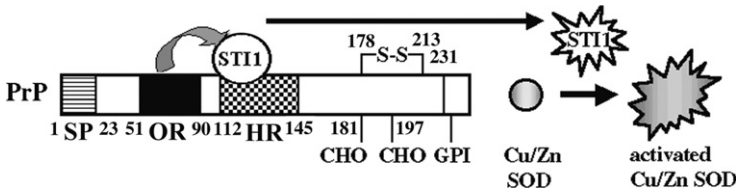


Fig. 5. Model of PrP^C-dependent SOD activation. After STI1 binds to N-terminal half of hydrophobic region (HR) on PrP^C, STI1 may be activated by octapeptide repeat region (OR), because both the OR and HR are indispensable for PrP(113–132) peptide inhibition (Fig. 4) and SOD activation [8]. PrP^C is localized to membrane, whereas STI1 to membrane and cytosol. Therefore, STI1 may be activated by PrP^C in membrane. The activated STI1 may shift to cytosol and activate Cu/Zn-SOD. The mechanisms of activation of STI1 by PrP^C or those of Cu/Zn-SOD by STI1 remain unknown. Copper might play an important role in the mechanisms of PrP^C-dependent activation of STI1 and Cu/Zn-SOD because: (1) activity of Cu/Zn-SOD is regulated by copper incorporation [23], and (2) copper enhances endocytosis of PrP^C [10]. PrP^C prevents apoptotic cell death at least in part by upregulating Cu/Zn-SOD activity [7].

PrP(113–132) peptide inhibition and anti-apoptotic function, suggesting that not only PrP^C–STI1 binding but also the presence of the OR of PrP^C is essential for STI1-mediated anti-apoptotic signals. The data are consistent with the hypothesis that after STI1 has bound to HR, STI1 is then activated by the OR to eventually mediate PrP-dependent SOD activation (Fig. 5). When animals are infected with prions, PrP^{Sc} is converted from PrP^C before being accumulated. The conversion of PrP^C to PrP^{Sc} leads to PrP^C deficiency [25], suggesting that not only a gain-of-function of PrP^{Sc} but also a loss-of-function of PrP^C contributes etiologically to induction of prion diseases. Our present studies suggest that susceptibility of $Prnp^{-/-}$ neuronal cells to cell death is probably due to reduced SOD activity induced by the absence of PrP^C–STI1 signals. In short, alteration of the signals mediated by PrP^C–STI1 binding may be due to another mechanism, whereby functional modification of PrP might have contributed to induction of prion diseases. Therefore, pharmacological stimulation of PrP^C–STI1 signals may serve as a useful approach in the treatment of prion disease.

Acknowledgments

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