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Nitric oxide induces prion protein via MEK and p38 MAPK signaling

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Abstract

The prion diseases or transmissible spongiform encephalopathy, such as human Creutzfeldt–Jakob disease (CJD) and so-called mad cow disease, are attributed to the causative agent, the scrapie variant of prion protein (PrP^{Sc}) which causes fatal neurodegeneration. To investigate if stresses such as nitric oxide (NO) induced the cellular isoform of prion protein (PrP^{C}), lipopolysaccharide, and sodium nitroprusside were used to treat N2a and NT2 cells, which resulted in elevated levels of the PRNP mRNA and prion protein. The signaling pathway for the NO-induced PrP^{C} production involved guanylyl cyclase, MEK, and p38 MAPK as shown by the effect of specific pharmacological inhibitors ODQ, PD98059, and SB203580, respectively. Knowing the PrP induction by the biologically existing stimulus, this study provides useful information about the possible cellular mechanism and strategies for the treatment of CJD.

Keywords: Nitric oxide; Prion protein; MEK; p38 MAPK

The prion protein (PrP) has been known as the responsible agent of the transmissible spongiform encephalopathies (TSE). In humans, these neurodegenerative diseases include sporadic or familial Creutzfeldt–Jakob disease (CJD), Gerstmann–Stäussler–Scheinker disease, fatal familial insomnia, kuru, and the variant CJD related to bovine spongiform encephalopathy. The manifestations of sporadic CJD include ataxia, myoclonus, and subacute mental and motor deterioration; and in brain the vacuolar or spongiform change and neuronal loss develop as the disease progresses [1]. People paid much attention to prion diseases due to the growing cases of mad cow disease and the variant CJD during the past years. The raising possibility of infectivity crossing the species barrier and different strains of infectious agent in animals gave evidences of "virion agents." But the more prevalent "protein-only hypothesis" had been proposed as the mechanism of TSE due to the accumulation of the infective scrapie variant, PrP^{Sc}. It is rich in β-structure, more radiation- and protease-resistant [2,3], with trimeric predisposition by the proposed parallel left-handed β-helical model [4], distinctive from the monomeric or dimeric α-helix-dominant PrP^{C} [5]. This pathogen, devoid of nucleic acid, is inclined to aggregate and causes different manifestations as infectious, genetic, or sporadic disorders. PrP^{Sc} propagation is probably related to post-translational modification and abnormal folding of the cellular form of prion protein (PrP^{C}). Infectivity depends on the presence of PrP^{C} in hosts and depletion of neuronal PrP in prion infection reversed the spongiosis in brain [6].

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The protein solely consisting of β-sheet-rich recombinant PrP was also infective and caused brain pathology [7]. Once exposed, PrP^{Sc} propagation develops in the subjects even during the latent or subclinical chronic stages [8].

The functions of the PrP may be related to the neural synaptic transmission, long-term potentiation, cell survival and neuroprotection, signal transduction, anti-oxidation and superoxide dismutase activity [9], neuroplasticity, epileptogenicity, and even playing a role in *Brucella* infection [10]. Some of the clinical manifestations of TSE/CJD can be explained by these disordered functions. As known there are stress molecules induced by PrP^{Sc} infection, involving heat shock and inflammation pathways [11]. In the past years, we had experienced two sporadic patients who initially had febrile events attributed to *Escherichia coli* infection, followed by full-blown CJD manifestations. The cell wall of *E. coli* has lipopolysaccharide (LPS), a common biological component inducing pro-inflammatory molecules and their following signals. Among these molecules we attempt to understand the relationship between nitric oxide (NO) and PrP. Physiologically NO may act as a signal to regulate gene expressions and the synaptic functions of the nervous system, but it also participates in neurodegeneration, such as Parkinson's disease (PD), a neurodegenerative disease involving the substantia nigra and striatum, as from the knowledge of the intranigral LPS injection into animal brains [12]. During PrP^{Sc} infection there were disordered oxidative stress responses, impaired metabolism of free radicals in neuronal cells [13], and poor production of NO in the microglia [14]. We suppose that the initial stress stimuli (such as LPS or/and NO) cause PrP induction, resulting in a "vulnerable stage" before PrP^{Sc} infection. Therefore, we applied LPS into the murine neuroblastoma N2a cells and the human carcinoma NT2 cell line, and found increased expression of PrP. Further nested works were conducted by sodium nitroprusside (SNP, one of the NO donors) treatment, resulting in induced expression of PrP and mRNA in NT2 cells. This induction pathway by NO was focused on guanylyl cyclase, MEK, and p38 MAPK, using their specific antagonists, resulting in decline in the expression level of PrP. These results may imply that stress-induced PrP production during the "vulnerable stage" occurs just before PrP^{Sc} invasion or/and propagation. The clinical application of blockade of the common signal pathways might be alternative clinical treatment of prion infections.

Methods

Cell culture. The N2a (Neuro-2a, ATCC number CCL-131, murine neuroblastoma cells) and NT2 (NTERA-2, ATCC number CRL-1973, a human carcinoma cell line with potency to differentiate into neuronal cells on induction) cells were cultured at 37 °C with 5% CO_2 supply in the presence of minimal essential medium (Eagle) and Dulbecco's modified Eagle's medium (Gibco-BRL Life Technologies), respectively, both supplemented with 10% calf serum. These two cell lines were seeded at a density of 1×10^6 with a recovery time of 18–20 h before the treatment of drugs.

Drug treatment. Lipopolysaccharide (LPS, from *E. coli* serotype 0128:B12, Sigma), sodium nitroprusside (SNP, Sigma; the NO donor), 1H-[1,2,4]oxaldiazolo-[4,3-a]quinoxalin-1-one (ODQ, the guanylyl cyclase inhibitor, Sigma), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059, the MEK inhibitor, Sigma), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580, the p38 MAPK inhibitor, Sigma) were administered at various concentrations as required.

Western blotting. The N2a cells received the 1.0 μg/ml LPS treatment for 4, 8, 12, 24, and 48 h. The human NT2 cells were treated with LPS at the concentration of 1.0 μg/ml for 12 h, and with SNP at 300 μM for 1, 3, 8, 12, and 24 h. The ODQ, PD98059, and SB203580 were co-administered with the 300 μM SNP, with a treatment duration of 8 h. The cells were scraped and homogenized in lysis buffer containing Hepes (pH 7.0), 0.5 M NaCl, 1 mM Na_3VO_4, Mini Protease Inhibitor Cocktail (1:100 from the stock, Roche), and 1% Triton X-100. Equivalent amounts of protein (100 μg) from the lysate were subjected to electrophoresis by using 4–12% Bis-Tris NuPAGE gels (Invitrogen), and proteins were electroblotted onto methanol-treated polyvinylidene difluoride membrane (Amersham-Pharmacia Biotech). After transfer, the membranes were blocked in 5% non-fat milk for 1 h and then hybridized with the specific primary antibodies, inducible nitric oxide synthase (1:1000, NOS2, Santa Cruz), and PrP (1:10,000, CD230, Serotec, for N2a cells; and 1:1000, sc-7693, Santa Cruz, for NT2 cells). The secondary antibodies of anti-mouse and anti-goat IgG-HRP (1:1000, Santa Cruz) were used for further hybridization, respectively. An enhanced chemiluminescence kit (Perkin-Elmer Life Sciences) was used for antibody-binding detection. Actin protein was examined as a reference (1:5000, Chemicon).

Reverse transcription and polymerase chain reaction (RT-PCR) of prion gene (PRNP). Initially the NT2 cells received LPS treatment at 10.0 and 1.0 μg/ml for 8 h or the SNP at 300 μM for 2 and 4 h. Total RNA from these cells was extracted by the addition of Trizol reagent (Zymest) followed by chloroform and precipitated by isopropyl alcohol. The RNA pellets were dissolved in water pre-treated with diethylpyrocarbonate (DEPC). Reverse transcription was performed by 5 μg of the RNA samples under the oligo-$d(T)_{15}$ priming in the presence of M-MLV reverse transcriptase (Promega) at 50 °C for 60 min. The PCR was conducted by the thermocycler (GeneAmp PCR system 2400, Perkin-Elmer) in the presence of *Taq* polymerase (Zymest) and the specific primer pairs (10 pmol/μl) (PRNP, sense: 5′-gaaccttggctgctggatg-3′ and anti-sense: 5′-acatctgctcaaccacgcg-3′; and the GAPDH, sense: 5′-gaaggtgaaggtcggagtc-3′ and anti-sense: 5′-caggaggcattgctgatga-3′). The temperature courses were 94 °C for 2 min; the first five cycles of 94 °C for 30 s, annealing at 58 °C (PRNP group) or 56 °C (GAPDH group) for 45 s, and 72 °C for 30 s; followed by 30 cycles (PRNP group) or 25 cycles (GAPDH group) of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s, and the final extension at 72 °C for 5 min, and soaked at 4 °C. The PCR products were subjected to electrophoresis onto 1% agarose gel and visualized by ethidium bromide staining.

Densitometric analysis. Photoshop7.0 (Adobe)-based histogram was used for the semi-quantitative densitometric analysis. In brief, the mean intensity and the total pixels timed each other, both derived from the histograms of the signal bands from the immunoblots or RT-PCR products. Data of the signal bands of the protein and RT-PCR products were at first standardized by those from the internal controls, actin and GAPDH, respectively, and then normalized by the ratios of control tests.

Results

We applied LPS treatment on a cell line model to evaluate the possible mechanism of prion diseases. The murine neuroblastoma N2a cells under LPS treatment had increased PrP (hybridized by anti-CD230 antibody) (Fig. 1A). There were three protein bands representing the non-, mono-, and di-glycosylated PrP with different molecular weights (19–37 kDa). All these three bands rose up during LPS treatment for more than 8 h. These cells also showed LPS-induced expression of NOS2 (Fig. 1B), as in the reported studies [14,15]. LPS treatment on the human NT2 cells increased the expression of PrP (Fig. 1C). The increased PrP level was associated with the increased level of mRNA of the prion gene (PRNP), prominent at 10.0 μg/ml LPS treated for 8 h (Fig. 1D). These implied the role of NO in the induction of mRNA and the protein levels of PrP during LPS stimulation in both murine and human cell lines. Due to the fact of NO production during stresses and neurodegeneration, we laid emphasis on this gas molecule and selected one NO donor, sodium nitroprusside (SNP), as the direct stimulator.

We used the human NT2 cells for the SNP treatment experiments. SNP at 100 and 300 μM caused little cell damage. The NT2 cells may express PrP constitutively but responded to SNP treatment by elevating the PrP (Fig. 2A), significantly on treatment for 8 and 12 h ($p < 0.05$, Fig. 2B). A decrementing tendency of this protein expression occurred at 12–24 h, as compared with that at 8 h, in NT2 cells. Although this increase is moderate, the induction effect is dose-dependent (at 100 and 300 μM SNP, Figs. 3A and B). The increase in protein level could be attributed to an elevated mRNA expression at 2 h after SNP treatment (Figs. 2C and D), the latter showing increment by about 1.5-fold as compared with the one without SNP treatment. These results suggested the role of NO released by SNP, causing induction of mRNA expression of PRNP and then of the PrP.

To confirm the relationship between NO and the induction of PrP expression, we evaluated the signaling pathway related to NO. The NO binds the guanylyl cyclase and increases the cytoplasmic concentration of cGMP which then phosphorylates the downstream signal proteins, including the MEK and p38 MAPK. We used the antagonists or blockers to evaluate the NO-related signaling pathways involving guanylyl cyclase (ODQ), MEK (PD98059), and p38 MAPK (SB203580). PrP expressions with the three individual inhibitors and co-administered 300 μM SNP declined (Fig. 3B), by about a half compared with the level on SNP treatment alone for 8 h. However, there was no significance among the reductions caused by these three inhibitors, but the expressions were lower than the mock control one without any SNP treatment. It could be attributed to the constitutive expression of PrP also involving these molecules. In brief, all these results suggest that the SNP releases NO, activating guanylyl cyclase, MEK, and p38 MAPK to induce PrP expression and the induced expression of PrP is reversed by the co-administered inhibitors.

Discussion

LPS causes the neurodegeneration, including the dopaminergic neurons demonstrated in intranigrally LPS-injected mice for the PD model [12,16], and interferes with the neurogenesis in hippocampus [17]. This biological molecule can explain the increased risk for PD in the rural residents and the unfiltered water consumer who had possibly exposed himself/herself to the

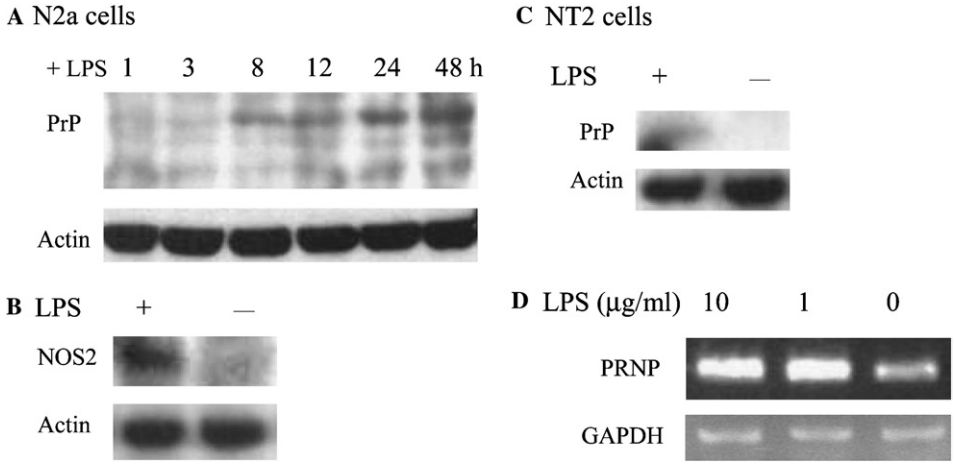

Fig. 1. LPS-induced expression of prion protein. (A) PrP expression was induced by LPS (1.0 μg/ml) in murine N2a cells. (B) LPS caused NOS2 induction in N2a cells after 12-h treatment. (C) PrP induction was also noted in human NT2 cells with LPS treatment at 1.0 μg/ml for 12 h. (D) The mRNA levels of prion gene (PRNP) in NT2 cells increased while LPS treatment (10.0 and 1.0 μg/ml) for 8 h.

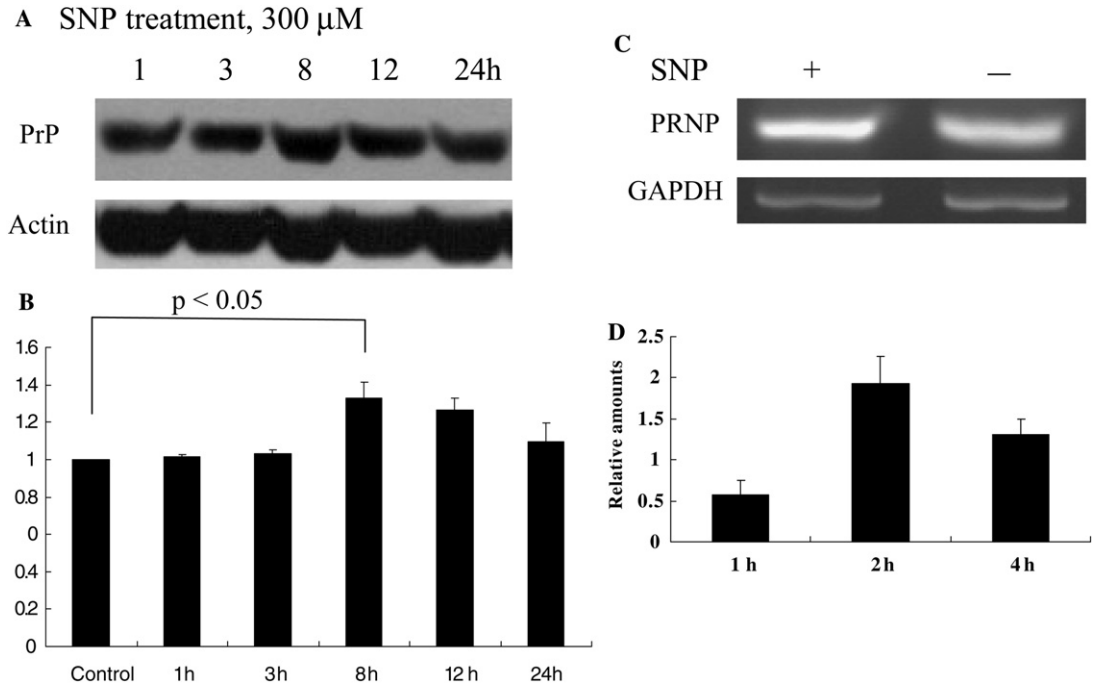

Fig. 2. Increased expression of PrP and PRNP mRNA induced by sodium nitroprusside. (A) The Western blots demonstrated the increased level of PrP after SNP treatment. Shown here is one representative experiment of three. (B) The expression by densitometric measurement increased under SNP treatment at 300 μM for 8 and 12 h ($p < 0.05$). (C) RT-PCR studies by specific primers revealed the increase in mRNA production of PRNP at 2 h after 300 μM SNP treatment. (D) The densitometry of the RT-PCR study showed an elevated mRNA level at 2 h and then a decrease at 4 h.

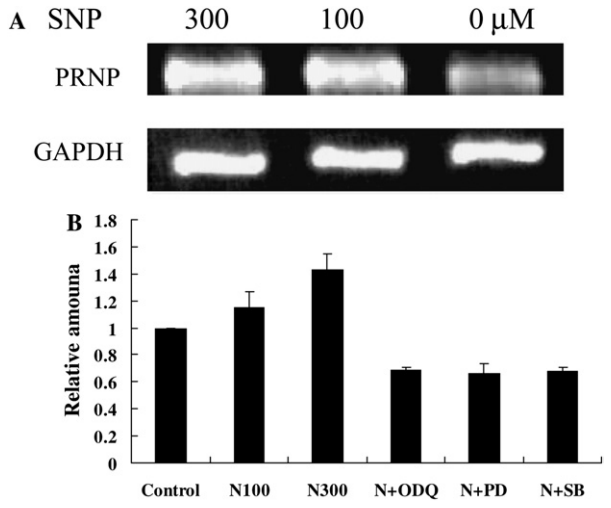

Fig. 3. Suppressed expression of PrP by various antagonists. (A) The 2-h expressions of PRNP mRNA induced by SNP were dose-dependent (SNP 100 and 300 μM). (B) The PrP expression induced by SNP (300 μM, N) for 8 h was reduced by the co-administered inhibitors, including ODQ (50 μM), PD98059 (10 μM, PD), and SB203580 (5 μM, SB), having significance as compared with that by SNP alone ($p < 0.05$).

Gram-negative bacteria during the early part of life [18,19]. It may render immune/inflammatory responses and also induce NO production. NO is a gas molecule that is produced by NO synthase, signaling via cGMP formation followed by the activation of MEK and p38 MAPK [20,21]. It is known that induction of NO associates with the neurodegeneration of PD and Alzheimer's disease [22]. These findings suggest a possible relationship between NO and CJD, the neurodegenerative disorder emphasized here. In this study, after the LPS and SNP treatment we found that the NO-induced PrP expression was through signaling of guanylyl cyclase, MEK, and p38 MAPK. This induction with the participation of NO is not regarded as an epiphenomenon during stresses due to the dose-dependent and time-related manners.

In addition to endogenous production, NO may also be derived from inorganic nitrate from diet [23]. No matter what the source of NO is, the induction of PrP by LPS and SNP in this study is one of the probable events during a "vulnerable stage," including the phenomena of the amount increasing, clustering or recruitment of PrP (Fig. 4), before the conformation change of PrP and the amyloid formation during the full-blown disease. Elevated level of the induced PrP^{C} might have a higher tendency toward the PrP^{Sc} formation spontaneously and alter the rate of propagation [24]. Prion propagation is one of the key issues in the pathogenesis of TSE/CJD. The protein conformation change might involve the non-catalytic/refolding or autocatalytic/seeding pathways, in spite of the cases with the transmissible or genetic diseases. Furthermore, evidences that in vitro lowering [25] or in vivo depletion of PrP^{C} [6] abrogated infection of PrP^{Sc} and reversed

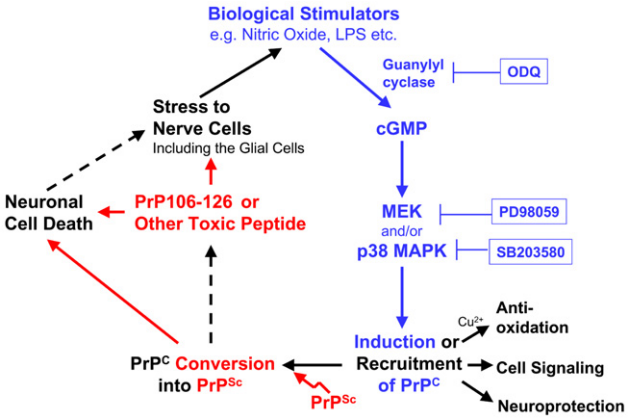

Fig. 4. A proposed mechanism showing the role of PrP upon induction and development of the TSE. The physiological induction of PrP by stresses (e.g., LPS and NO) with signaling by MEK and p38 MAPK may be self-protecting by anti-oxidation, cell survival signaling, and other neuroprotective pathways during the "vulnerable stage" (the blue way). But on prion infections there is already enough amount of PrP to increase the rapidity of PrP^{Sc} propagation with conformation change, followed by neuronal cell death (the red way). Toxic peptide may also play a role. Cell-dying stresses may have the further effect on induction.

brain pathology cast emphasis on the key role of the presence and amount of PrP^{C} in prion diseases. On the other hand, the story of new proteins produced by NO [26] may also be true for the PrP^{C} for the purpose of self-protection against the oxidative stress by its activities of anti-oxidation and superoxide dismutase [9]. Recent evidence of PrP^{C} clustering associated with phosphorylation of the extracellular receptor kinases 1 and 2 [27] supported our proposition that induced PrP^{C} may trigger survival signals before infection.

NO can also be produced by the PrP fragment, such as $PrP_{106–126}$ [28]. It is unknown whether $PrP_{106–126}$ exists by endogenous proteolysis or with abnormal expression in diseased animals. Yet, the cleaved PrP indeed occurred by the reactive oxygen species (e.g., H_2O_2), phorbol ester, and the bacterial enzymes. $PrP_{106–126}$ can cause the aggregation of PrP and induce protease-resistant prions. It is known as a "toxic peptide" rendering cell death [29], as the case of the proteolytic peptide C31 from the amyloid precursor protein [30].

Dr. Stanley Prusiner raised the protein-only hypothesis of PrP propagation in 1982 [31]. Although PrP^{C} and PrP^{Sc} have the same molecular weight and amino acid sequences, PrP^{Sc} has the main features of amyloidogenicity and protease resistance. In the microenvironment Mn-bound PrP^{C}, instead of the copper binding, may result in resistance to protease and compromised anti-oxidation [32], implying some clinical significance during TSE/CJD. The lymphoid dendritic cells play a role on neuro-invasion in the transmission of prion diseases [33], and explain the pathogenesis of the subjects with new variant CJD who took the beef from the mad cows. Besides, it is possible that extracellular shedding and accumulation of PrP^{Sc} are present in the TSE/CJD brains due to the intercellular transfer or shedding of the PrP [34,35]. This transfer explained the work by Jeffrey et al. [36] who found the extracellular accumulation of PrP^{Sc} from the prion-expressing astrocytes and the damage of the prion-null neurons in a transgenic mouse model. More works are needed to know how PrP^{Sc} is transferred during the prion diseases and the resulting death signaling in neurons.

Induction of neuronal PrP possibly occurs during the "vulnerable stage," mediated by NO, MEK, p38 MAPK, or/and other molecules from the activated glial cells. The induction is a physiological phenomenon, initially for the purposes of self-protection by the anti-oxidation, cell survival signaling, and other pathways. Once reaching the amount threshold of the induced PrP with one invaded molecule of PrP^{Sc}, the cells might deteriorate into a pathological condition including enhanced rapidity of PrP^{Sc} propagation, increased lipid peroxidation, and impairment of free radical metabolism [13], followed by neuronal cell death (Fig. 4). NO may play one of the cardinal roles in this pathogenesis. Our work is of prime importance to evaluate the early mechanism of prion diseases. Lowering of NO production and blockade of the signal pathway may be the possible ways for the early treatment of the hyper-acute stage of prion infections.

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