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p75^{NTR} activation of NF-κB is involved in PrP106-126-induced apoptosis in mouse neuroblastoma cells

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Abstract

Neuronal death is a pathological hallmark of prion diseases. Synthetic prion peptide PrP106-126 can convert PrP^{C} into protease-resistant aggregates, which can cause neurotoxicity *in vivo* and *in vitro*. Various cell surface proteins can participate in the infection process of prions. p75^{NTR} can interact with PrP106-126 and has a neurotoxic effect on neurons. However, for p75^{NTR} lacking intrinsic catalytic activity domain in cytoplasm, p75^{NTR} -associated signaling molecular and the signaling events in cytoplasm in p75^{NTR}-mediated apoptosis responding to PrP106-126 remain still unknown. Thus p75^{NTR} -associated NF-κB signaling pathway was investigated in this study. Herein PrP106-126-induced apoptosis in mouse neuroblastoma cell line N2a, PrP106-126 significantly up-regulated p75^{NTR} expression on mRNA and protein levels. For the first time we found that PrP106-126 induced activation of NF-κB by Western blot assay, and blocking the interaction of p75^{NTR} with PrP106-126 by p75^{NTR} polyclonal antibody sc-6189 or pretreatment with inhibitor NF-κB SN50 reduced the activation of NF-κB and attenuated the apoptotic effect by PrP106-126. This study offers a possible interpretation that NF-κB signaling pathway was activated by the interaction of PrP106-126 with p75^{NTR}, and NF-κB activity showed the pro-apoptotic effect in PrP106-126-induced apoptosis in N2a cells. Involvement of NF-κB signaling pathway in p75^{NTR}-mediated apoptosis may partially account for the PrP106-126-induced neurotoxicity in N2a cells.

Keywords: TSE; PrP106-126; p75^{NTR}; NF-κB; Apoptosis

1. Introduction

Transmissible spongiform encephalopathies (TSEs), also termed as prion diseases, comprise a group of neurodegenerative disorders in which spongiosis, vacuolation, neuronal loss, astrocytosis and microglial activation are common pathological events of the central nervous system (CNS) (Prusiner, 1991). The central event in pathogenesis of prion diseases is the formation of a protease-resistant misfolded protein named PrP^{Sc}, which is a conformationally modified normal cellular PrP denoted PrP^{C} (Cohen and Prusiner, 1998). Functional expression of PrP^{C} is essential for propagating prion, and mice devoid of functional PrP genes are unable to propagate prion agent (Sailer et al., 1994). Other cell surface proteins besides PrP^{C} were reported to be necessary for developing disease.

The mouse neuroblastoma cell line N2a, which is capable of propagating prion proteins (Butler et al., 1988), has been widely used in cell culture systems to study TSEs. A synthetic peptide homologous to human PrP region 106-126 (PrP106-126) shares many physicochemical features with PrP^{Sc}, including the propensity to form β-sheet-rich, insoluble, and protease-resistant fibrils (Forloni et al., 1993; Selvaggini et al., 1993; Brown, 2000). The neurotoxicity of PrP106-126 mainly depends on the expression of endogenous PrP^{C} and the induction of the aggregation and internalization of cell surface PrP^{C} (Gu et al., 2002). PrP106-126 is neurotoxic towards primary cultures of rat hippocampal neurons, cortical neurons and cerebellar cells, and has been used as a model to study prion diseases *in vitro* (Forloni et al., 1993; Brown et al., 1996; Ning et al., 2005). However, the *vitro* mechanisms of PrP106-126-induced neuronal death remain to be fully elucidated.

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The p75^{NTR} receptor, a member of tumor necrosis factor (TNF) receptor superfamily (Locksley et al., 2001), functions as a receptor for PrP106-126 and is related to apoptosis through the binding of the prion peptide to p75^{NTR} (Lee et al., 2003). p75^{NTR}, locating within caveolae-like domains of cell (Bilderback et al., 1997), lacks intrinsic catalytic activity in cytoplasm. However, the p75^{NTR} cytoplasmic tail contains several potential motifs for interactions with downstream signaling molecules. The NF-κB transcription factor chiefly resides in the cytoplasm. Upon extracellular stimulation, p65 subunit of NF-κB rapidly translocates into the nucleus and activates gene expression. p75^{NTR} is required for NF-κB signaling pathway. Translocation of NF-κB is absent in Schwann cells isolated from p75$^{NTR-/-}$ mice (Dechant and Barde, 1997). In several neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), NF-κB activity was significantly elevated in the neurons of brains of patient (Kaltschmidt et al., 1997; Hunot et al., 1997). In human neuroblastoma cells, the binding of β-amyloid to p75^{NTR} was reported to activate NF-κB and result in apoptosis (Kuner et al., 1998). NF-κB activation has been involved in gene regulation of cellular processes like inflammation, immune response, cell proliferation and apoptosis (Chen et al., 2002). However, in prion peptide PrP106-126-induced apoptosis in N2a cells, whether the interaction of PrP106-126 with p75^{NTR} activates NF-κB signaling and the functional role of NF-κB translocation remain uncertain. In this study, we found that PrP106-126 interacted with p75^{NTR} and induced translocation of NF-κB, which promoted the apoptotic effect on N2a cells by PrP106-126. Our results provided the first evidence that p75^{NTR}-mediated apoptosis by PrP106-126 in N2a cells partially occurs through NF-κB signaling pathway.

2. Materials and methods

2.1. Materials

The human sequence of the prion protein fragment (PrP106-126) (KTNMKHMAGAAAAGAVVGGLG) and the scrambled sequence (SCR) were synthesized by Shanghai Sangon (China). Lyophilized peptides were dissolved in PBS (pH 7.4) at a concentration of 5 mM and stored at −20 °C as stock solutions. NF-κB SN50 (BIOMOL Research Laboratories Inc. Plymouth Meeting, PA), an inhibitor peptide of NF-κB nuclear translocation, was used at 18 μM (Lin et al., 1995). Goat polyclonal antibody sc-6189 (Santa Cruz Biotechnology) against a peptide mapping at the amino terminus of p75^{NTR} was used in competition analysis. Rabbit polyclonal anti- NF-κB p65 and anti-p75^{NTR} antibodies were from Beyotime Institute of Biotechnology (China) and Boisynthesis Biotechnology (China), respectively. Mouse monoclonal anti β-actin, anti-mouse and anti-rabbit horseradish peroxidase-conjugated goat antiserums were from Zhongshan Goldenbridge Biotechnology (China).

2.2. Cell culture

Mouse neuroblastoma cells N2a (ATCC CCL-131^{TM}) were cultured in DMEM/F12 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO), standard antibiotics (100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 °C in 5% CO_2. After 24 h culture, the medium was replaced with fresh DMEM/F12 medium containing 5% FBS and standard antibiotics. Then freshly prepared PrP106-126, SCR and NF-κB SN50 or polyclonal antibody sc-6189 were applied.

2.3. Quantitative RT-PCR of p75^{NTR}

Total RNA was extracted from 6×10^6 cells treated or untreated using a TRIZOL RNA Extraction Kit (Invitrogen, Austria). The concentrations of RNA from different groups were determined by spectrophotometry (BioPhotometer Eppendorf; Germany) and quantified by measuring the absorbance at OD_{260}. Equal amounts of RNA were subjected to the same reaction of reverse transcription-polymerase chain reaction (RT-PCR) with DNA Synthesis Kit (Promega, USA). For p75^{NTR}, the forward primer was 5′-CTGTGTGGAGG-CAGACGATG-3′ and the reverse primer was 5′-GAATGAGGTTGT-CAGCGGTG-3′, annealing temperature was 55 °C, and PCR products were 464bp (from nucleotide 443–906, Genbank: NM033217) (Fig. 1A). For β-actin, an endogenous house keeping gene, the forward primer was 5′-TGCTGTCCCTGTATGCCTCTG -3′ and the reverse primer was 5′-TTGATGTCACGCACGATTTCC-3′, annealing temperature was 60 °C, and PCR products were 223bp (from nucleotide 495 to 717, Genbank: NM007393)

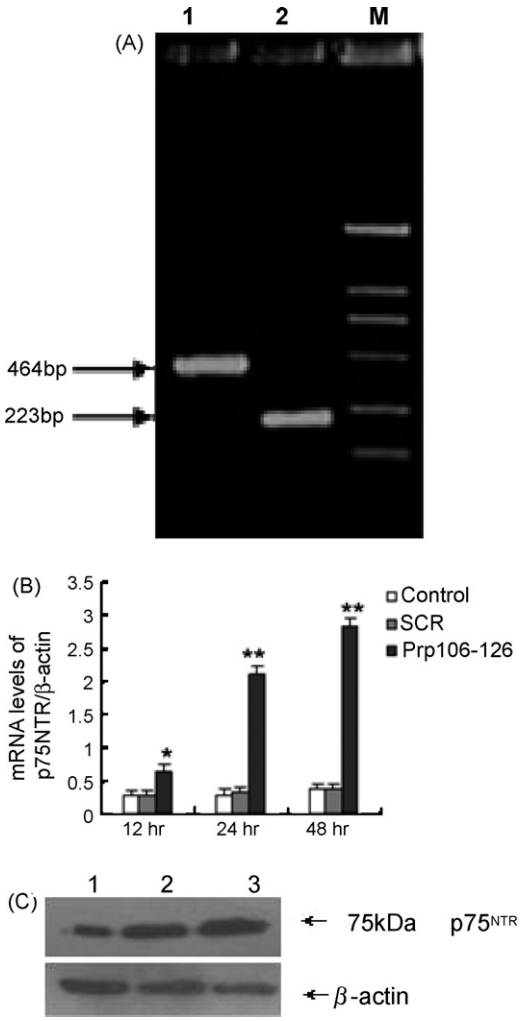

Fig. 1. Effect of PrP106-126 on mRNA and protein expression of p75^{NTR}. (A) RT-PCR products for p75^{NTR} (lane 1) and β-actin (lane 2). M, DNA marker (DL2000 plus). (B) The relative mRNA levels of p75^{NTR} gene in N2a cells treated with PrP106-126 (25 μM) or SCR (25 μM) for 12, 24, and 48 h by quantitative RT-PCR. The data represent the relative level of p75^{NTR}. The values are indicated as means ± S.D. of triplicate experiments. $*P < 0.05$ and $**P < 0.01$, statistically significant differences between PrP106-126-treated groups and untreated control. (C) The relative protein levels of p75^{NTR} in N2a cells treated with PrP106-126 (25 μM) for 24 h and 48 h by western blot analysis. Lane 1, untreated control; lane 2, 25 μM PrP106-126 for 24 h; lane 3, 25 μM PrP106-126 for 48 h. P75^{NTR} expression was normalized to β-actin. Data are representative of three independent experiments.

(Fig. 1A). For standard curve preparation, the plasmid DNA of $p75^{NTR}$ and β-actin were constructed, respectively. Equal amounts of cDNA were then subject to quantitative RT-PCR with DNA Engine OpticonTM2 continuous fluorescence detection system and SYBR® GreenIqPCR kit (TIANGEN Bio-Tech, Beijing, China). The expression level of $p75^{NTR}$ gene was determined by normalizing the copies of $p75^{NTR}$ to that of β-actin using a relative standard curve method.

2.4. Western blot of $p75^{NTR}$

Total protein was extracted from 6×10^6 cells treated or untreated with PrP106-126 using Protein Extraction Kit (Applygen Technologies Inc, Beijing, China). The protein concentrations were quantified by measuring the absorbance at OD_{280} and equal amounts of total protein were subjected to SDS-PAGE. After transfer onto PVDF membranes, proteins were immunoblotted with a mouse monoclonal anti β-actin antibody (1:1000) or a rabbit polyclonal anti-$p75^{NTR}$ antibody (1:500). Then membranes were probed with an anti-mouse or an anti-rabbit horseradish peroxidase-conjugated goat antiserum (1:5000). Protein bands were visualized by ECL procedure.

2.5. DNA fragmentation assay

N2a cells were cultured as described above for different treatments. After stimulation, 6×10^6 cells were lysed and DNA was extracted and purified with the Apoptotic DNA Ladder Kit (Beyotime Institute of Biotechnology, China) according to manufacturer's instructions. Equal amounts of purified apoptotic DNA were applied to electrophoresis on a 1.5% agarose gel at 45 V (1.5 h, RT). After staining with 1 mg/ml of ethidium bromide, DNA bands were visualized by a UV light and photographed.

2.6. Cell proliferation assay

N2a cells were grown (1×10^3) in 96-well plates. After different treatments, the cells were further incubated with MTT (0.5 mg/ml) at 37 °C for 4 h followed by addition of 150 μl of DMSO. The absorbance values were measured at 490 nm using microplate reader (BIO-RAD, USA). The cell viability was plotted as percent of control.

2.7. Nuclear extract and Western blot

N2a cells were stimulated and nuclei proteins were extracted using Nuclear Extract kit (Applygen Technologies Inc., Beijing, China). Nuclei protein concentrations were quantified by measuring the absorbance at OD_{280} and equal amounts of protein were analyzed by SDS-PAGE followed by Western Blot analysis with a mouse anti β-actin monoclonal antibody (1:1000) or a rabbit anti-p65 polyclonal antibody (1:2000), and then anti-mouse or anti-rabbit horseradish peroxidase-conjugated goat antiserum (1:5000). Protein bands were visualized by ECL procedure.

2.8. Statistical analysis

Comparison of treatment effects was carried out using one-way analysis of variance techniques of SPSS 12.0. Data were expressed as means ± S.D. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Increased expression of $p75^{NTR}$ on mRNA and protein levels

N2a cells were treated with 25 μM of freshly prepared PrP106-126 for 12, 24, and 48 h. As shown in Fig. 1B, the expression of $p75^{NTR}$ gene in N2a cells was increased in a time-dependent manner. Compared with untreated control, the increase of mRNA expression of $p75^{NTR}$ occurred after 12 h treatment. A 7-fold and 7.8-fold increase was seen after 24 h and 48 h treatment, respectively ($P < 0.01$). No significant changes were observed in any SCR-treated groups. The increased expression of $p75^{NTR}$ was also obviously seen on protein level. Fig. 1C showed that 24 h or 48 h treatment with 25 μM PrP106-126 increased the protein expression of $p75^{NTR}$ in a time-dependent manner. This effect for 12 h treatment was slight (data not shown). Upregulation of $p75^{NTR}$ expression on mRNA and protein levels was seen within 12–48 h. It indicated that PrP106-126 activated $p75^{NTR}$ on N2a cells.

3.2. $p75^{NTR}$-mediated apoptotic DNA and cell proliferation

Apoptotic DNA ladder method was employed to detect the apoptotic effect on N2a cells. As shown in Fig. 2A, formation of apoptotic bodies was significant in PrP106-126-treated group in

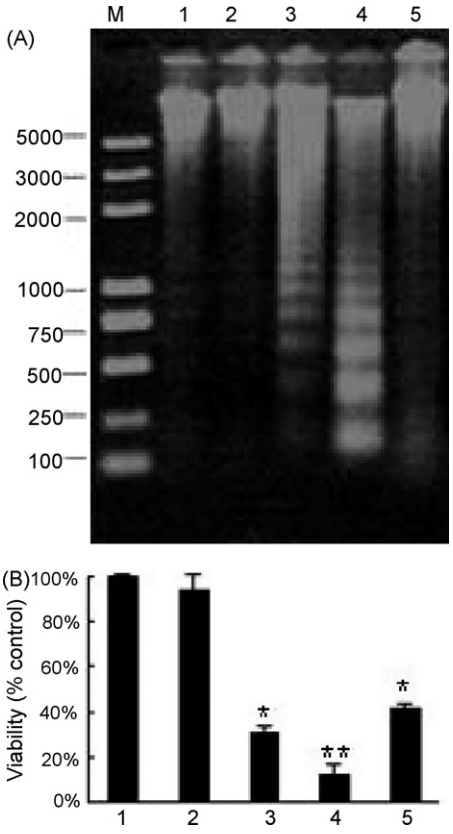

Fig. 2. Apoptotic DNA ladder and cell proliferation induced by PrP106-126 on N2a cells. (A) Apoptosis of N2a cells treated for 24 h or 48 h with 25 μM PrP106-126 in the absence or presence of 10 μg/ml anti-$p75^{NTR}$ antibody sc-6189 by DNA ladder assay. Lane 1, control; lane 2, SCR; lane 3, with 25 μM PrP106-126 for 24 h; lane 4, with 25 μM PrP106-126 for 48 h; lane 5, with 10 μg/ml anti-$p75^{NTR}$ antibody sc-6189 and 25 μM PrP106-126 for 24 h; M, DNA ladder marker. Data are representative of three independent experiments. (B) Cell viability of N2a cells treated for 24 h or 48 h with 25 μM PrP106-126 in the absence or presence of 10 μg/ml anti-$p75^{NTR}$ antibody sc-6189 by MTT assay. Lane 1, control; lane 2, SCR; lane 3, with 25 μM PrP106-126 for 24 h; lane 4, with 25 μM PrP106-126 for 48 h; lane 5, with 10 μg/ml anti-$p75^{NTR}$ antibody sc-6189 and 25 μM PrP106-126 for 24 h; The data are indicated as means ± S.D. of triplicate experiments and plotted as percent of control. *$P < 0.05$, statistically significant differences between treated groups and untreated control.

a time-dependent manner, with higher apoptotic DNA concentration after 48 h treatment than 24 h treatment. Co-treatment for 24 h with 10 μg/ml anti-p75^{NTR} antibody sc-6189 and 25 μM PrP106-126 attenuated apoptotic bodies in N2a cells. No DNA ladder was observed either in untreated control or SCR-treated group. We further confirmed the PrP106-126-induced cell proliferation by MTT assay. Fig. 2B showed that the cell viability in PrP106-126-treated group also decreased in a time-dependent manner, from 30.8% for 24 h to 11.6% for 48 h. However, the cell viability increased in the presence of 10 μg/ml anti-p75^{NTR} antibody sc-6189 compared with PrP106-126-treated alone for 24 h, increasing from 30.8% to 43.0% (lane 3 and 5, respectively). No significant change was observed in SCR-treated group. Anti-p75^{NTR} antibody partially inhibited the cell apoptosis. These results indicated that PrP106-126 induced significant apoptosis within 24–48 h, and this effect was partially mediated by the cell surface receptor p75^{NTR}.

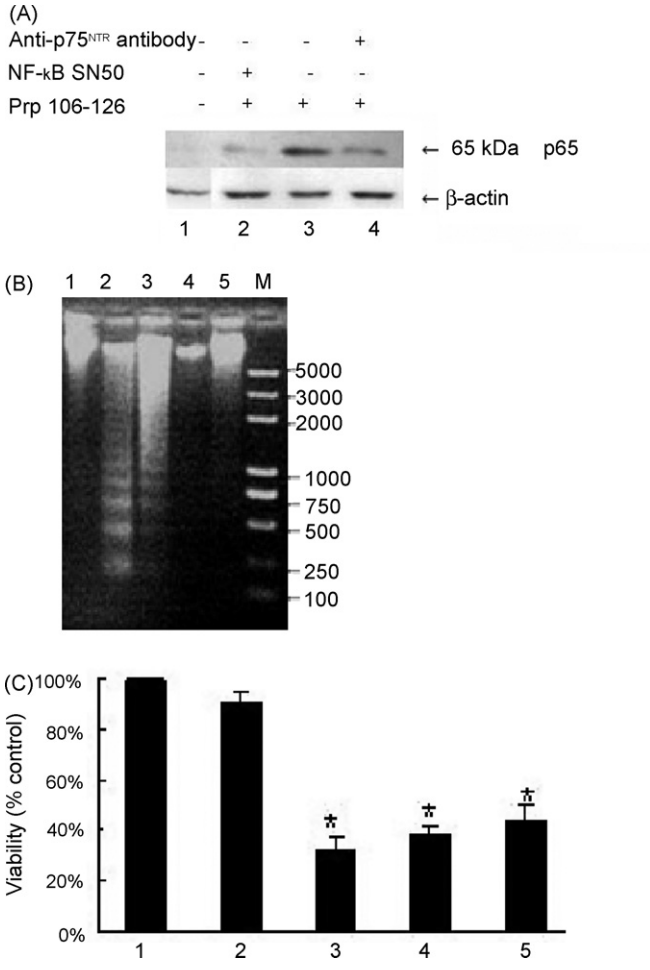

Fig. 3. Nuclear translocation of p65 and apoptosis or cell proliferation induced by PrP106-126 in N2a cells. (A) Nuclear translocation of p65 by western blot analysis. P65 expression was normalized to β-actin. Lane 1, untreated control; lane 2, treatment with 25 μM PrP106-126 for 30 min with pretreatment with 9 μM NF-κB SN50 for 30 min; lane 3, treatment for 30 min with 25 μM PrP106-126; lane 4, co-treatment for 30 min with 25 μM PrP106-126 and 10 μg/ml anti-p75^{NTR} antibody sc-6189. (B) DNA ladder analysis of N2a cells treated for 24 h with 25 μM PrP106-126 alone (lane 2) or with pretreatment with 9 μM NF-κB SN50 for 30 min (lane 3); lane 5, co-treatment for 24 h with 10 μg/ml anti-p75^{NTR} antibody sc-6189 and 25 μM PrP106-126; lane 1, 9 μM NF-κB SN50 for 24 h; lane 4, untreated control. Data are representative of triplicate experiments. (C) Cell viability of N2a cells treated for 24 h with 25 μM PrP106-126 alone (lane 3) or with pretreatment with 9 μM NF-κB SN50 for 30 min (lane 4); lane 5, co-treatment for 24 h with 25 μM PrP106-126 and 10 μg/ml anti-p75^{NTR} antibody sc-6189; lane 1, untreated control; lane 2, 9 μM NF-κB SN50; The data are indicated as means ± S.D. of triplicate experiments and plotted as percent of control. *$P < 0.05$, statistically significant differences between treated groups and untreated control.

3.3. Nuclear translocation of p65 and its apoptosis and cell proliferation effect

As shown in Fig. 3A, no p65 was detected in the nuclei in untreated control cells. Stimulation for 30 min with 25 μM PrP106-126 induced p65 translocation and aggregation in nuclei of N2a cells. However, 25 μM PrP106-126 for 30 min with pretreatment with 9 μM NF-κB SN50 for 30 min (lane 2) or co-treatment for 30 min with PrP106-126 and 10 μg/ml anti-p75^{NTR} antibody sc-6189 (lane 4) decreased density of p65 band. For apoptotic DNA ladder assay (Fig. 3B), treatment with 25 μM PrP106-126 for 24 h induced significant DNA ladders in N2a cells, whereas pretreatment with NF-κB SN50 for 30 min or co-treatment with anti-p75^{NTR} antibody and PrP106-126 for 24 h reduced the apoptotic effect. The cell proliferation analysis (Fig. 3C) showed the identical results as apoptotic DNA ladder assay. The cell viability was increased with NF-κB SN50 (38.1%) or anti-p75^{NTR} antibody (44.2%) compared with 25 μM PrP106-126-treated alone (31.9%). 9 μM NF-κB SN50 alone had no effect on N2a cells. No significant changes were observed in any SCR-treated groups (data not shown). NF-κB activation was seen at 30 min after 25 μM PrP106-126 treatment, and inhibiting of NF-κB activation could partially block apoptosis. In PrP106-126 treated N2a cells, the competitive interaction of anti-p75^{NTR} antibody with p75^{NTR} decreased NF-κB activation and partially blocked the cell apoptosis.

4. Discussion

Previous report has indicated that p75^{NTR} promoted apoptosis in p75^{NTR}-transfected neuroblastoma cell lines (Bunone et al., 1997). Our results also demonstrated that, p75^{NTR} expression on mRNA and protein levels was significantly elevated in N2a cells when exposed to PrP106-126 within 24–48 h, and increased expression of p75^{NTR} was accompanied by significant apoptosis. Increased expression of p75^{NTR} revealed that PrP106-126 activated and interacted with p75^{NTR}. Goat polyclonal antibody sc-6189 raised against the N-terminus of p75^{NTR} partially blocked the apoptosis on N2a cells by PrP106-126, through competition for the interaction with p75^{NTR}, which was consistent with the results that the specific binding of PrP106-126 to p75^{NTR} could be inhibited by antibody sc-6189 in SK-N-BE neuroblastoma cells (Della-Bianca et al., 2001). NF-κB subunit p65 was also found to translocate to the nuclei of N2a cells by western blot analysis, and the translocation occurred transiently within 30 min treatment. Inhibition of NF-κB translocation with NF-κB SN50 partially blocked the apoptosis, suggesting that NF-κB activity played an apoptotic effect on N2a cells. p75^{NTR}

antibody sc-6189 obviously decreased NF-κB activation and cell apoptosis, indicating that inhibiting the interaction of PrP106-126 with the cell surface receptor $p75^{NTR}$ could either inhibit NF-κB activation or partially block the apoptosis. The observation suggested that NF-κB signaling pathway was activated in $p75^{NTR}$-mediated neurotoxicity and had pro-apoptotic effect on N2a cells in response to PrP106-126.

In vivo study had suggested that NF-κB activity was significantly increased in the brains of Scrapie-infected mice (Kim et al., 1999). In this *in vitro* study, we also proved that prion peptide PrP106-126 could activate NF-κB and NF-κB activation induced apoptosis of N2a cells. 25 μM PrP106-126 activated NF-κB signaling pathway in human monocyte-derived dendritic cells within 15 min by electrophoretic mobility shift assay, resulting in differentiation and activation of DCs (Bacot et al., 2003). We demonstrated that 25 μM PrP106-126 activated NF-κB within 30 min by western blot, resulting in apoptosis in N2a cells. The time difference and the pro-apoptotic or pro-survival effect of NF-κB translocation might depend on cell type and upstream signaling molecules of the cells.

In this study, $p75^{NTR}$ antibody sc-6189 and NF-κB inhibitor NF-κB SN50 were both found to decrease N2a cell apoptosis induced by PrP106-126. However, the decreasing effect was higher in $p75^{NTR}$ antibody treatment, with lower apoptotic DNA concentration (Fig. 3B) or higher cell viability (Fig. 3C) in anti-$p75^{NTR}$ antibody treatment. This poses the problem that $p75^{NTR}$-dependent c-Jun N-terminal kinase (JNK) signaling pathway might be activated in N2a cells and result in apoptosis besides NF-κB signaling pathway, but JNK activation correlated with PrP106-126 or prion remains to be investigated.

$p75^{NTR}$-activated NF-κB signaling pathway was biologically important for PrP106-126-induced apoptosis in N2a cells. PrP106-126-induced neurotoxicity was mainly PrP^{C}-dependent, and $p75^{NTR}$ and its NF-κB signaling pathway played an assistant role for PrP^{C} on apoptotic effect in N2a cells, probably through regulating PrP^{C} conformational transition or facilitate the internalization of β-sheet-rich fibrils.

In summary, this study had demonstrated that NF-κB signaling pathway was activated by the interaction of PrP106-126 with $p75^{NTR}$, and NF-κB activity showed the pro-apoptotic effect in PrP106-126-induced apoptosis in N2a cells. PrP106-126 significantly increased $p75^{NTR}$ expression on mRNA and protein levels. $p75^{NTR}$ antibody sc-6189 could inhibit NF-κB activation and thus partially block apoptosis of N2a cells through the competitive interaction of $p75^{NTR}$. NF-κB SN50 could inhibit NF-κB activation and partially block apoptosis of N2a cells. The results presented herein obtained by using the prion model of PrP106-126 should be further clarified using PrP^{Sc}, and these data should be further confirmed by gene-knockdown experiment, concerning $p75^{NTR}$ antibody sc-6189 and NF-κB SN50 being potential therapeutics.

Acknowledgments

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