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Original Article

PP2 and piceatannol inhibit $PrP_{106-126}$-induced iNOS activation mediated by CD36 in BV2 microglia

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Prion diseases are a group of transmissible fatal neurodegenerative disorders of humans and animals, including bovine spongiform encephalopathy, scrapie, and Creutzfeldt–Jakob disease. Microglia, the resident macrophages of the central nervous system, are exquisitely sensitive to pathological tissue alterations, altering their morphology and phenotype to adopt a so-called activated state and perform immunological functions in response to pathophysiological brain insults. Although recent findings have provided valuable insights into the role microglia play in the proinflammatory events observed in prion, the intracellular signaling molecules responsible for the initiation of these responses remain to be elucidated. It seems that microglial activation involve $PrP_{106-126}$ binding and the activation of cell surface immune and adhesion molecules such as CD36 and integrins, with the subsequent recruitment of Src family tyrosine kinases such as Fyn, Lyn, and Syk kinases. In the present study, we show that CD36 is involved in $PrP_{106-126}$-induced microglial activation and that PP2 and piceatannol (Pic) can abrogate neurotoxic prion peptides-induced inducible nitric oxide synthase activation in microglia. These findings unveil a previously unrecognized role of PP2 and Pic as Src family kinase Fyn and the tyrosine kinase Syk inhibitor involved in neurotoxic prion peptides–microglia interactions, thus providing new insights into mechanisms underlying the activation of microglia by neurotoxic prion peptides.

Keywords cd36; pp2; piceatannol (Pic); $PrP_{106-126}$; bv2 microglia; iNOS

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Introduction

Prion diseases are a group of transmissible fatal neurodegenerative disorders of humans and animals, including bovine spongiform encephalopathy in cattle, scrapie in sheep, and Creutzfeldt–Jakob disease in humans. They are caused by the conversion of cellular prion protein (PrP^{C}) into the pathological isoform (PrP^{Sc}) through conformational changes [1,2]. Neuropathological features of prion diseases include neuronal vacuolation, neuronal loss, astrogliosis, and accumulation of activated microglial cells in affected brain areas [3]. Neurotoxic prion protein (PrP) fragment 106–126 ($PrP_{106-126}$) is commonly used as a model for the investigation of PrP^{Sc} neurotoxicity, as it possesses similar physicochemical and pathological properties to PrP^{Sc}. It forms amyloid fibrils with high β-sheet content, shows partial proteinase K resistance, and is neurotoxic *in vitro* [4].

Microglia, the resident macrophages of the central nervous system (CNS) parenchyma, are exquisitely sensitive to pathological tissue alterations, altering their morphology and phenotype to adopt a so-called activated state and perform immunological functions in response to pathophysiological brain insults [5,6]. A wealth of data have demonstrated that microglia have very diverse effector functions, in line with macrophage populations in other organs [7]. Increasing evidence has also indicated that microglial activation contributes to neuronal damage in several neurodegenerative diseases including Alzheimer's disease, prion diseases, Parkinson's disease, multiple sclerosis, and Huntington's disease [6,8]. In prion diseases and other neurodegenerative disorders, microglia becomes over-activated and releases reactive oxygen species (ROS), NO, and cytokines, which might cause vascular damage in addition to neurodegeneration [8–10]. Scavenger receptors which are pattern recognition receptors expressed on the microglial surface seem to associate physically to form a receptor complex, which is one of the primary and common pathways through which diverse toxin signals are transduced into ROS production in microglia [9].

Although recent findings have provided valuable insights into the role microglia play in the proinflammatory events

observed in prion diseases, the intracellular signaling molecules responsible for the initiation of these responses remain to be elucidated. CD36, a class B scavenger receptor, is an 88-kDa membrane glycoprotein expressed on platelets, monocytes, and macrophages. CD36 and several other cell type scavenger receptors represent a family of cell surface glycosylated proteins that bind with a wide variety of ligands [11]. CD36 is expressed on the surface of microglia and is involved in microglia-mediated immune response in the CNS [12,13]. The role of CD36 in the amyloid-beta (Aβ)-induced microglial activation in Alzheimer's disease has been extensively investigated [14,15], and some researches have proved that CD36 participates in $PrP_{106-126}$-induced activation of microglia [16], but the mechanism is not clear. The signaling pathways downstream of CD36 involve the ligand-dependent recruitment and activation of non-receptor tyrosine kinases Src family (Fyn and Lyn), the serine/threonine kinase MAPK family (JNK and p38 MAPK), the specific mitogen-activated protein kinases (MAPKs), and the Vav family of guanine nucleotide exchange factors; and the generation of intracellular ROS [11].

It has been suggested that microglial activation involves $PrP_{106-126}$ binding and the activation of cell surface immune and adhesion molecules such as CD45, CD40, CD36, and integrins, with the subsequent recruitment of Src family tyrosine kinases such as Fyn, Lyn, and Syk kinases [15]. The extracellular signal-regulated kinase (ERK)/MAPK pathways are then activated, which induces proinflammatory gene expression and leads to the production of cytokines and chemokines [17]. In the present study, we investigated how CD36 participates in the $PrP_{106-126}$-induced microglial activation by mediating inducible nitric oxide synthase (iNOS), and the proinflammatory cytokines up-regulation. Our results suggested that signaling pathways downstream of CD36 involve non-receptor tyrosine kinases Src family kinases fyn and syk kinases. Of clinical relevance, exploring the components of these pathways may yield novel targets for therapeutic modulation and help to develop drugs for prion diseases.

Materials and Methods

Materials

CD36 antibody was purchased from Gene Tex (Irvine, USA) and rabbit anti-mouse iNOS antibody and rabbit anti-mouse β-actin antibody from Bioworld Technology (St Louis Park, USA). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody was from Beyotime Biotechnology (Shanghai, China), PP2 (AG 1879; 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) from Merck KgaA (Darmstadt, Germany), and piceatannol (3,3′,4,5′-tetrahydroxystilbene, Pic) and lipopolysaccharides (LPS) from Sigma (St Louis, USA). BV2, a murine microglial cell line, was kindly provided by Dr Yuqin Liu (Cell Culture Center, Xiehe Medical University, Beijing, China).

PrP peptide

PrP peptides $PrP_{106-126}$ (sequences KTNMKHMAGAAA AGAVVGGLG and AVGMHAGKGLANTAKAGAMVG), were synthesized by Sangon Bio-Tech (Shanghai, China). The purity of prion peptides was >95% according to the data from synthesizer. The peptides were dissolved in 0.1 M phosphate-buffered saline (PBS) to a concentration of 1 mM, and left to aggregate at 37°C for 12 h. Experiments were conducted with the final peptide concentration of 100 μM.

Cell culture and PrP peptide treatment

BV2 microglial cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Waltham, USA). The cells were maintained in a humidified incubator with 5% CO_2 at 37°C. The BV2 cells were pretreated with or without 1 μg/ml of CD36 antibody for 1 h, and then treated with 100 μM of aggregated peptide $PrP_{106-126}$ or 300 ng/ml LPS in culture medium for 6 and 12 h. The BV2 cells were incubated with or without the indicated concentrations of the inhibitors, PP2 (10 μM) and Pic (25 μg/ml), or both for 30 min at 37°C, and then cultured with aggregated peptide $PrP_{106-126}$ (100 μM) or LPS (300 ng/ml) in the culture medium for 6 and 12 h. The cell treatment was performed in triplicate.

Quantification of iNOS, interleukin-1β, tumor necrosis factor-α, and β-actin RNA expression

Total RNA was extracted from the cells using Direct-zol™ RNA MiniPrep kit (ZYMO Research, Orange County, USA), and reverse-transcribed into cDNA using a commercial cDNA synthesis kit (Fermentas, Glen Burnie, USA) using oligo(dT) 18 primers in accordance with manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed using SYBR Green I Master Mix (Bio-Rad, Hercules, USA) and a thermal cycler (DNA Engine Opticon™ 2 system; MJ Research, Waltham, USA) with the primers listed in **Table 1**. The amplification efficiency of these primers had been established by means of calibration curves. The total volume for qPCR was 20 μl, consisting of 8 μl of water, 0.5 μl of each primer (10 μM), 10 μl of Master Mix, and 30 ng of cDNA. The PCR cycles were denaturation at 94°C for 2 min, 40 cycles of 94°C for 5 s, 55°C for 20 s, and 72°C for 30 s, followed by 1 cycle at 72°C for 10 min appended for a single fluorescence measurement above the melting temperature of possible primer–dimers. Finally, a melting step was performed consisting of 10 s at 70°C and slow heating at a rate of 0.1°C/s up to 95°C with continuous fluorescence measurement. Quantification was performed using the comparative CT method ($2^{-\Delta\Delta CT}$). All samples were analyzed in triplicate.

Table 1 Primers used for quantitative PCR in this study

Gene	Primer sequence (5′→3′)	PCR product size (bp)	Annealing temperature (°C)
β-actin	Forward: TGCTGTCCCTGTATGCCTCTG	223	60
	Reverse: TTGATGTACCGCACGATTTCC		
iNOS	Forward: TGTGTCAGCCCTCAGAGTAC	312	57
	Reverse: CACTGACACTYCGCACAA		
IL-1β	Forward: GCCTCAAAGGAAAGAATCTA	229	4
	Reverse: AAACAGTCCAGCCCATACTT		
TNF-α	Forward: GGCGGTGCCTATGTCTCA	221	62
	Reverse: CACTTGGTGGTTTGCTACG		

Immunoblotting of CD36 and iNOS

BV2 microglial cells were washed with PBS on ice. Cellular proteins were extracted using a protein extraction kit (Boster Biotechnology Inc., Wuhan, China). Equal amounts of protein (40 μg in each lane) were separated electrophoretically using 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and then the proteins on the gel were transferred onto 0.45-μm polyvinylidene fluoride (Millipore, Bedford, USA). The membranes were soaked in blocking buffer (5% skimmed milk in TBS-T solution, pH 7.4) at 37°C for 1 h, and then incubated overnight at 4°C with primary anti-CD36 (1 : 500) and anti-iNOS (1 : 500) antibodies. The membranes were washed with TBS-T (pH 7.5, containing 10 mM Tris, 0.15 M NaCl, 0.05% Tween-20), and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1 : 5000). Bands of immunoreactive protein were visualized after incubation with enhanced chemifluorescence reagent for 5 min on an image system (Versadoc; Bio-Rad). The blot was stripped and reprobed with anti-β-actin antibody (for cytoplasmic extracts) to estimate the total amount of loaded protein.

Statistical analysis

All assays were performed in triplicate. Data were expressed as mean ± SD. All comparisons for parametric data were made using Student's *t*-test or one-way analysis of variance followed by *post hoc* Turkey's test using the SPSS software (version 13.0, SPSS Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

$PrP_{106-126}$-induced up-regulation of iNOS production in microglia

We tested whether neurotoxic $PrP_{106-126}$ engagement of microglia led to microglial activation. Western blot analysis showed that after neurotoxic $PrP_{106-126}$ stimulation in BV2 cells, iNOS protein was observed. Both LPS (used as positive control) and $PrP_{106-126}$ increased the expression of iNOS at 6 and 12 h [**Fig. 1(A)**]. The band intensity of western blots was assessed by densitometry [**Fig. 1(B)**]. The production of iNOS at 12th hour was nearly more than twice, when compared with that at 6th hour [**Fig. 1(B)**]. These findings were consistent with our previous findings that $PrP_{106-126}$ peptide stimulates the microglial activation [16,18,19].

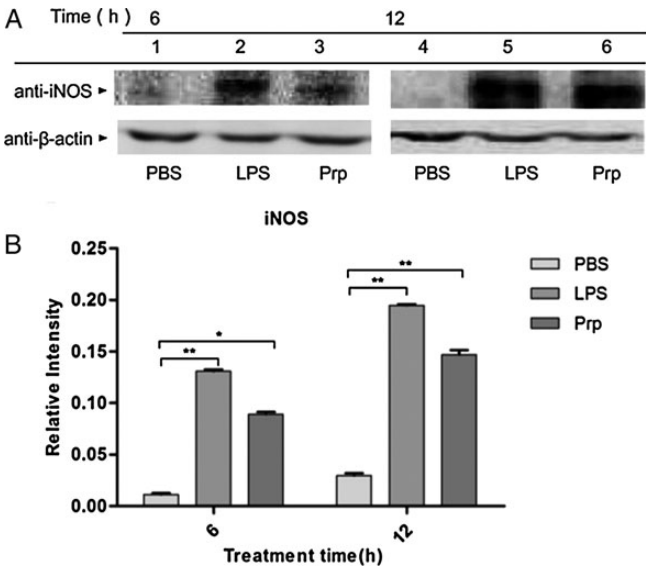

Figure 1 $PrP_{106-126}$-induced up-regulation of iNOS production in microglia at 6th and 12th hour BV2 microglia cells were incubated with PBS, LPS (300 ng/ml), or $PrP_{106-126}$ (100 μM) for 6 or 12 h. Proteins were prepared and separated on SDS–polyacrylamide gels and immunoblotted with antibodies to iNOS and β-actin, as described in 'Materials and Methods' section. (A) Representative blots of iNOS and actin. (B) Band intensity of iNOS normalized to β-actin. $*P < 0.05$ and $**P < 0.01$ compared with PBS control.

$PrP_{106-126}$ induced an increase of CD36 protein levels in microglia

The identification of cell surface molecules which mediate the PrP synthetic peptides interaction with microglia is of great importance, as it represents the first point of intervention in the events leading to the pathophysiology of prion

diseases. We have recently shown that $PrP_{106-126}$ induces an increase of CD36 mRNA levels in BV2 microglia cells [16,20]. To further identify the participation of CD36 in $PrP_{106-126}$-induced microglial activation, the protein level of CD36 was measured in microglia stimulated by LPS or $PrP_{106-126}$ for 6 and 12 h. We found that both LPS and neurotoxic $PrP_{106-126}$ increased the expression of CD36 at 6th and 12th hour, when compared with negative control (**Fig. 2**). The expression of CD36 in cells stimulated by $PrP_{106-126}$ slightly fell from 6 to 12 h, meanwhile, the CD36 level in the cells exposed to LPS increased. Taken together, these results suggested that microglia might employ the cell surface receptor CD36 to detect and respond to PrP synthetic peptides and other stimuli.

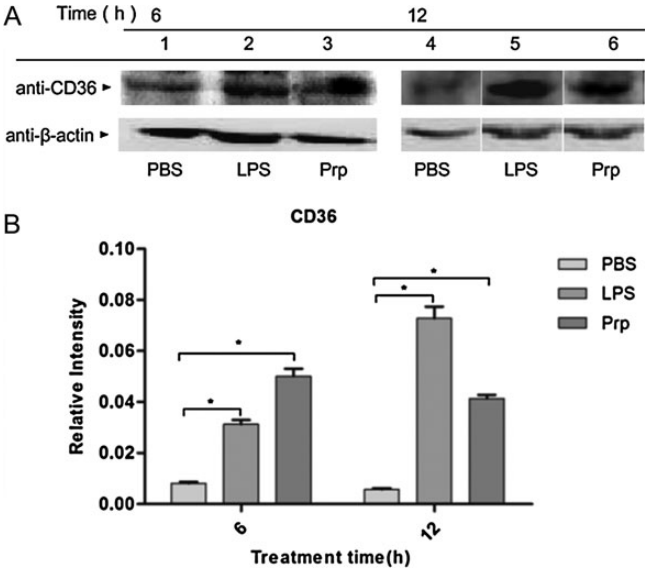

Figure 2 $PrP_{106-126}$ induced an increase of CD36 protein levels in microglia at 6th and 12th hour BV2 cells were incubated with PBS, LPS (300 ng/ml), or $PrP_{106-126}$ (100 μM) for 6 or 12 h. Proteins were prepared and separated on SDS–polyacrylamide gels and immunoblotted with antibodies to CD36 and β-actin, as described in 'Materials and Methods' section. (A) Representative blots of iNOS and actin. (B) Band intensity of iNOS normalized to β-actin. $*P < 0.05$ and $**P < 0.01$ compared with PBS control.

$PrP_{106-126}$ induced a CD36-dependent up-regulation of iNOS production

In light of our findings that CD36 expression was increased in response to $PrP_{106-126}$ stimulation, we hypothesized that CD36 might be a necessary cell surface receptor in the intracellular signaling pathway leading to iNOS production. To examine the role of CD36 in the process of $PrP_{106-126}$--stimulated microglial activation, CD36 monoclonal antibody was used to block CD36 and inhibit the formation of the receptor complex. We examined the level of iNOS in the protein extract from BV2 microglia exposed to $PrP_{106-126}$ with or without pre-incubation with anti-CD36 antibody. Western blot analysis showed that $PrP1_{06-126}$ resulted in the up-regulation of iNOS in BV2 (**Fig. 3**). Pretreatment with anti-CD36 antibody followed by $PrP_{106-126}$ stimulation abolished the $PrP_{106-126}$-induced iNOS up-regulation. Pre-incubation with CD36 antibody did not affect LPS (positive control)-stimulated iNOS expression in the 6-h assay, but decreased the up-regulation of iNOS by LPS after treatment for 12 h.

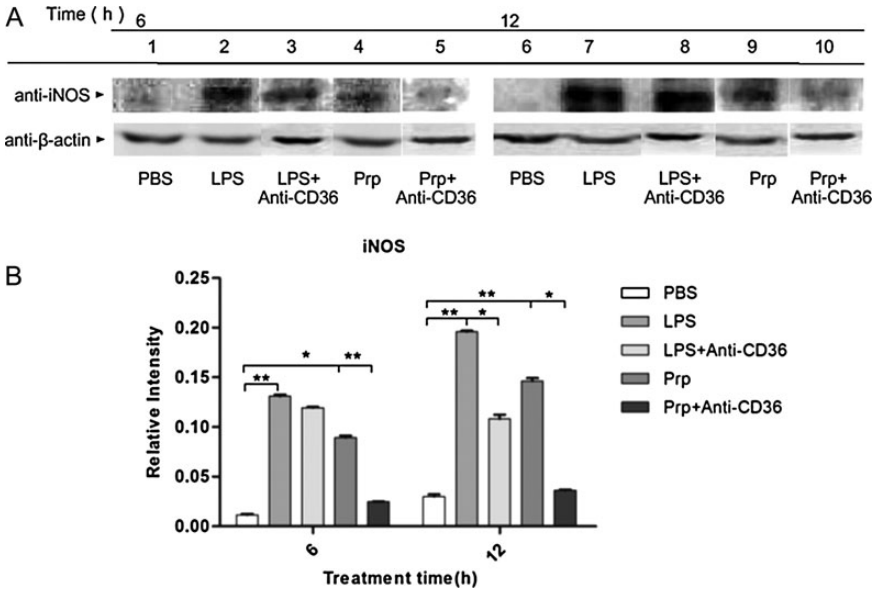

Figure 3 The blockade of CD36 abolished $PrP_{106-126}$-induced iNOS in BV2 cells for 6 and 12 h BV2 cells were exposed to PBS, LPS (300 ng/ml), or $PrP_{106-126}$ (100 μM) with or without pre-incubation with CD36 antibody (1 μg/ml). Proteins were prepared and separated on SDS–polyacrylamide gels and immunoblotted with antibodies to iNOS and β-actin, as described in 'Materials and Methods' section. (A) Representative blots of iNOS and actin. (B) Band intensity of iNOS normalized to β-actin. $*P < 0.05$ and $**P < 0.01$ compared with PBS control.

PP2 and Pic inhibited production of $PrP_{106-126}$-induced iNOS

Microglia and THP-1 (a human leukemic cell line) cells respond to fibrillar β-amyloid (fAβ) by activating the Src family kinase Fyn as well as the tyrosine kinase Syk, which have been previously reported to be required for proximal signaling events in the microglial response to β-amyloid fibrils [21–23]. Binding of fibrils to microglia was mediated by CD36-associated receptor complex. The types of Aβ and $PrP_{106-126}$ fibrillar amyloid deposits share an invariant association with reactive glial cells, particularly microglia [22]. There is abundant evidence of a microglial-derived inflammatory component in both diseases [22]. Interestingly, both PP2, a specific inhibitor of src family kinases [24], and Pic, a Syk-selective inhibitor [25], have been shown to decrease the production of iNOS in response to a variety of stimuli [26,27]. Therefore, we sought to determine whether PP2 and Pic could inhibit the production of iNOS following stimulation with $PrP_{106-126}$.

The iNOS expression in response to LPS and $PrP_{106-126}$ and the kinase inhibitors was analyzed by qPCR. Treatment of BV2 cells for 6 and 12 h with LPS or $PrP_{106-126}$ led to a significant increase in iNOS mRNA level, when compared with control cells treated with PBS, in which the level of iNOS was negligible [**Fig. 4(A–D)**, columns 1, 5]. PP2, Pic, or the combination of PP2 and Pic resulted in a significant decrease of iNOS mRNA expression in both LPS and $PrP_{106-126}$-treated cells [**Fig. 4(A–D)**, columns 5–8]. However, the iNOS level in the group which was treated with LPS and PP2 for 12 h was nearly equal to that in the positive control group (LPS treatment). Furthermore, the extent of iNOS decrease at 12th hour was much smaller than that at 6th hour for the groups treated with $PrP_{106-126}$, which was opposite to the groups treated with LPS.

To elucidate the effect of PP2 and Pic on signaling, we analyzed the iNOS expression in response to LPS or $PrP_{106-126}$ by western blotting. Results showed that the iNOS expression was undetectable in untreated cells, but

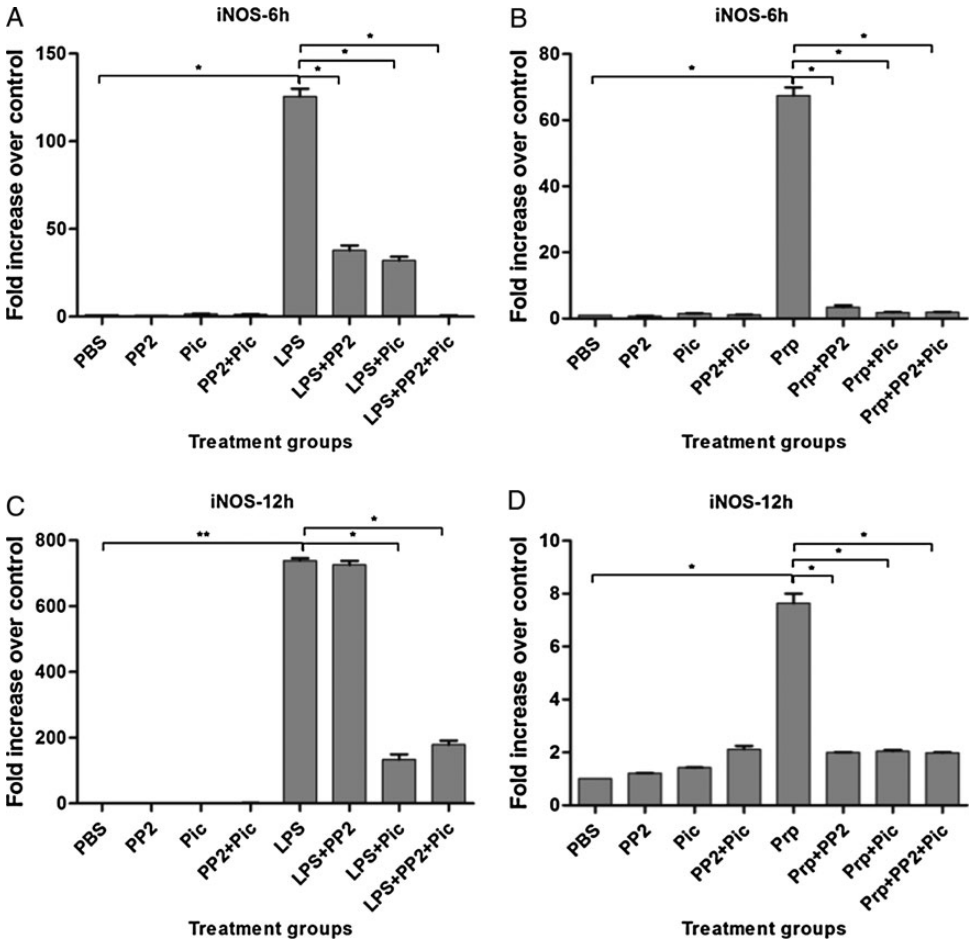

Figure 4 The mRNA expression of iNOS, in BV2 microglia treated with $PrP_{106-126}$ in the presence or absence of PP2 or Pic BV2 cells were incubated with or without the inhibitors, PP2 (10 μM), Pic (25 μg/ml), or PP2 and Pic together (10 μM and 25 μg/ml, respectively) for 30 min at 37°C, and then cultured with aggregated peptide $PrP_{106-126}$ (100 μM) or LPS (300 ng/ml) for 6 or 12 h. Total mRNA was isolated and reverse-transcribed. The mRNA levels of iNOS were measured by quantitative reverse-transcribed PCR (qRT-PCR). The mRNA level of iNOS was expressed as fold increase over control cells which were exposed to PBS only. *$P < 0.05$ and **$P < 0.01$ compared with control (incubated without LPS or $PrP_{106-126}$).

was clearly induced by LPS and $PrP_{106-126}$ [**Fig. 5(A–C)**, lanes 1, 5, and 9]. Treatment with the inhibitors alone had little effect on iNOS expression [**Fig. 5(A–C)**, lanes 2–4, columns 2–4]. Co-treatment of cells with LPS and $PrP_{106-126}$ together with the respective inhibitor for 6 h showed that the LPS-induced iNOS expression was decreased by the combination of PP2 and Pic by almost 5 folds when compared with LPS stimulus alone [**Fig. 5(A,B)**, lanes 5–8] and that $PrP_{106-126}$-induced iNOS expression was reduced by PP2, Pic, or PP2 and Pic by 2–5 folds compared with $PrP_{106-126}$ stimulus alone [**Fig. 5(A,C)**, lanes 5–12]. The trend of each group with LPS or $PrP_{106-126}$ treatment for 12 h was similar to the corresponding group of 6 h [**Fig. 5(D–F)**], except that the iNOS expression in the cells treated with LPS and PP2 decreased at 12th hour compared with that at 6th hour [**Fig. 5(D,E)**, lane 7, column 7]. Moreover, the iNOS expressions of $PrP_{106-126}$ and inhibitors groups at 12th

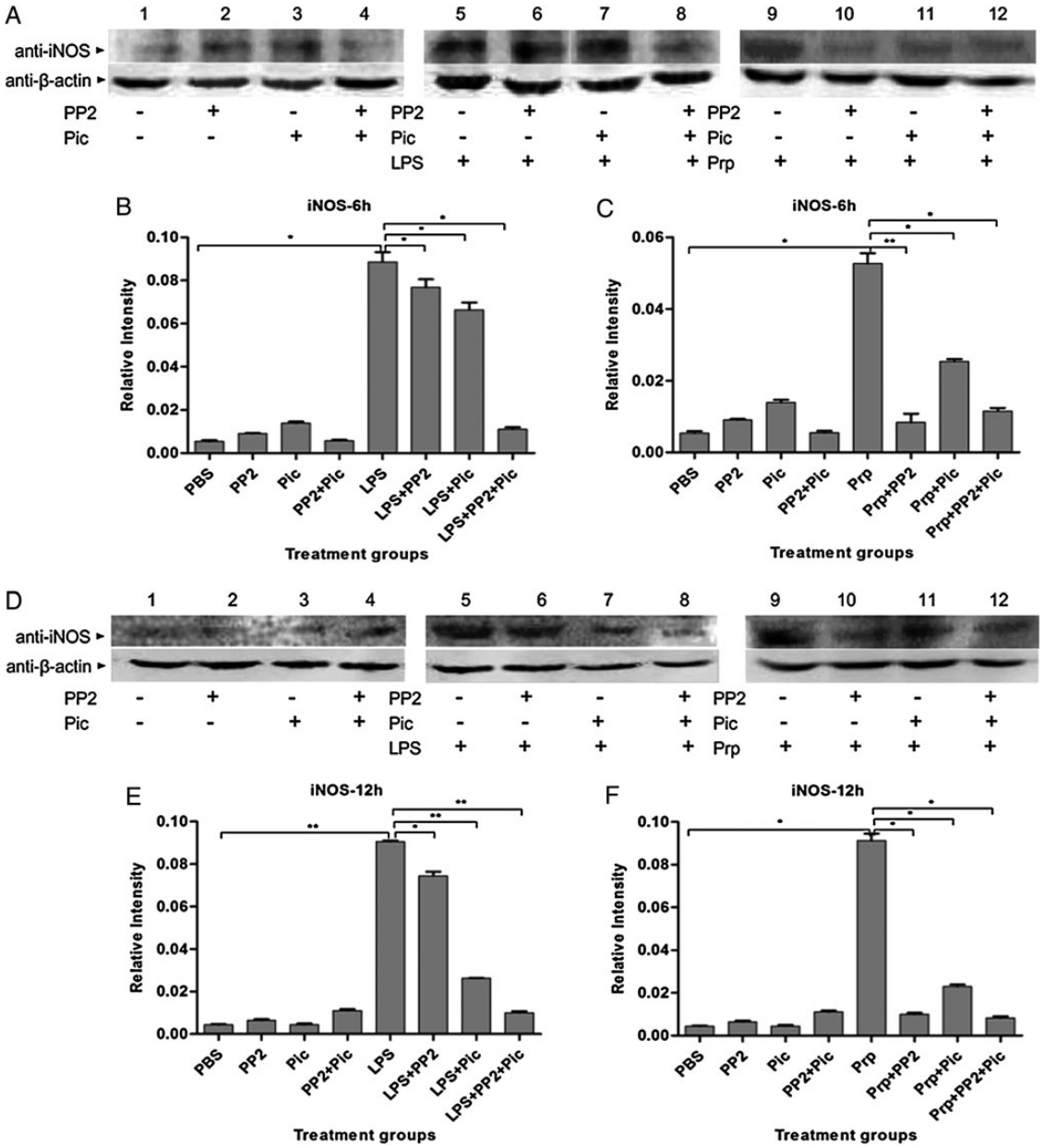

Figure 5 PP2 and Pic abolished $PrP_{106-126}$-induced iNOS in BV2 cells at 6th hour (A–C) and 12th hour (D–F) BV2 cells were incubated with or without the inhibitors, PP2 (10 μM), Pic (25 μg/ml), or PP2 and Pic together (10 μM and 25 μg/ml, respectively) for 30 min at 37°C, and then cultured with aggregated peptide $PrP_{106-126}$ (100 μM) or LPS (300 ng/ml) for 6 or 12 h. Proteins were prepared and separated on SDS–polyacrylamide gels and immunoblotted with antibodies to iNOS and β-actin, as described in 'Materials and Methods' section. (A, D) Representative blots of iNOS and actin were shown. (B, C, E, and F) Bars represent the relative levels of iNOS, compared with β-actin, and were expressed as arbitrary units. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with PBS control.

hour were higher than those at 6th hour [**Fig. 5(C,F)**, columns 5–8]. These results suggested that PP2 and Pic could inhibit the production of $PrP_{106-126}$-induced iNOS.

$PrP_{106-126}$-induced increase of proinflammatory cytokines, interleukin-1β, and tumor necrosis factor-α mRNA expression in microglia were inhibited by PP2 or Pic

qPCR analysis showed that the pretreatment of BV2 cells with kinase inhibitors PP2 and Pic down-regulated the $PrP_{106-126}$- or LPS-induced interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mRNA expression (**Fig. 6**). PP2 or Pic did not affect TNF-α or IL-1β expression, but reduced the $PrP_{106-126}$-stimulated increase in TNF-α and IL-1β expression. The up-regulation of TNF-α and IL-1β induced by LPS was also reduced by these two inhibitors, with a greater effect by Pic than by PP2. These two inhibitors showed no synergistic or additive activities.

Discussion

Previous studies have shown that pathological PrP can activate microglia and induce the release of inflammatory cytokines and chemokines *in vivo* and *in vitro* [28,29]. Furthermore, the cell surface receptor CD36 has been shown to be an important factor in prion disease-associated microglial activation and release of inflammatory cytokines and chemokines [16]. Although it is clear that PrP^{Sc}

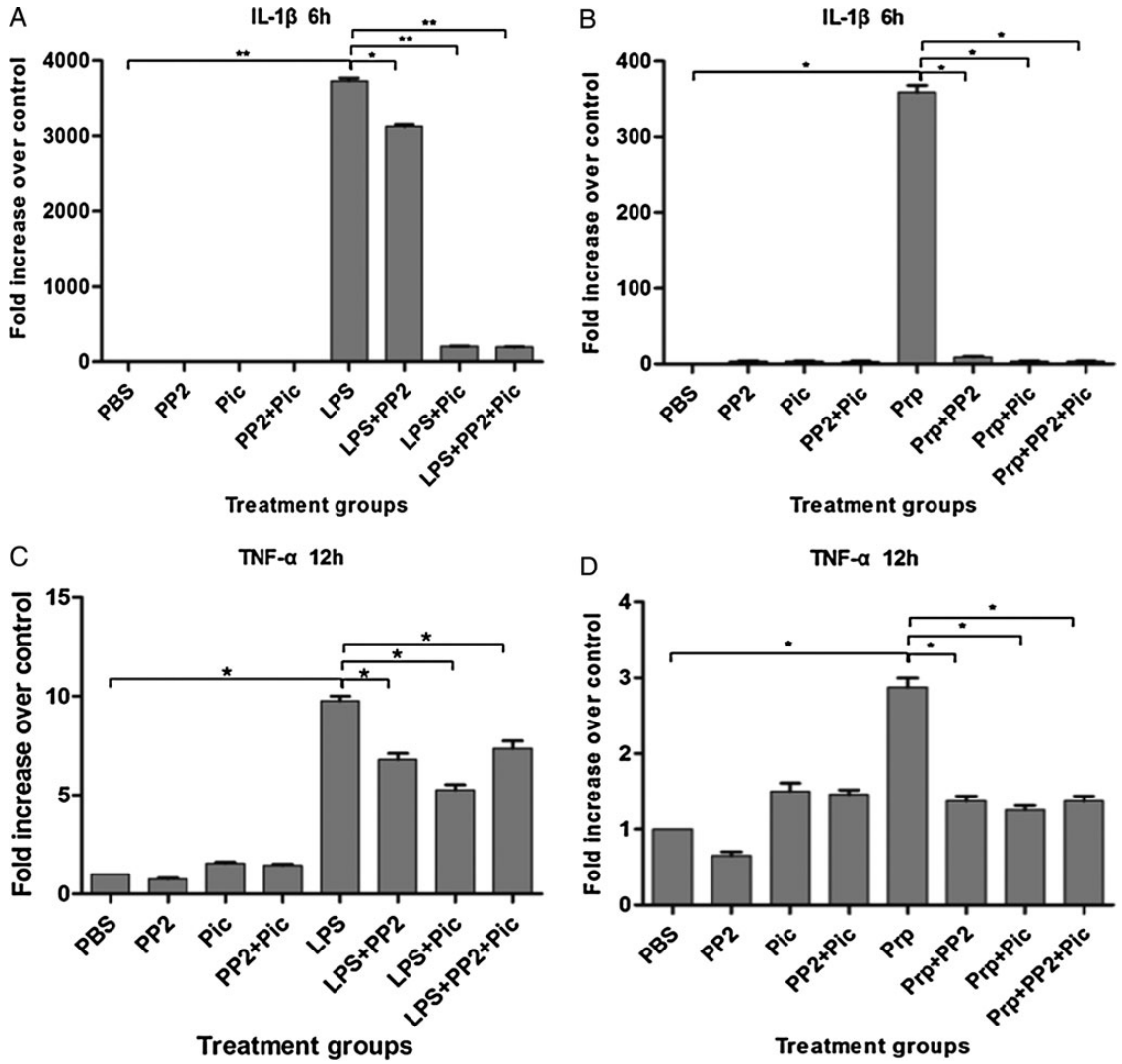

Figure 6 The mRNA expression of proinflammatory cytokines IL-1β at 6th hour (A, B) and TNF-α at 12th hour (C, D) in BV2 microglia treated with $PrP_{106-126}$ in the presence or absence of PP2 or Pic BV2 cells were incubated with or without the inhibitors, PP2 (10 μM), Pic (25 μg/ml), or both PP2 and Pic (10 μM and 25 μg/ml, respectively) for 30 min at 37°C, and then cultured with aggregated peptide $PrP_{106-126}$ (100 μM) or LPS (300 ng/ml) for 6 or 12 h. Total mRNA was isolated and reverse-transcribed. The mRNA levels of proinflammatory cytokines were measured by qRT-PCR. The mRNA level of each cytokine was expressed as fold increase over control cells which were exposed to PBS only. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control (incubated without LPS or $PrP_{106-126}$).

induces the production of iNOS and some inflammatory cytokines and chemokines, the mechanism of prion-mediated processing and release of iNOS is unclear.

We extend our previous work by identifying new elements which participate in the $PrP_{106-126}$-induced signaling cascades. We started by examining the effect of exposure to $PrP_{106-126}$ and LPS on the production of iNOS in BV2 microglia cells. The iNOS protein increased significantly after 6 h of exposure to prion peptide and steadily increased over 12 h. The general pattern of the iNOS production observed in this study was similar to what we have reported in recent studies [16,19,30] and a related report [30], which indicated that $PrP_{106-126}$ induce the activation of iNOS in microglia.

The identification of cell surface molecules, which mediate the PrP peptide interaction with microglia, is of great importance as it represents the first point of intervention in the events leading to the pathophysiology of prion diseases. We have recently shown that $PrP_{106-126}$ induces an increase in CD36 mRNA level in BV2 microglia [16,20]. To further identify the participation of CD36 in $PrP_{106-126}$-induced microglial activation, we tested the protein level of CD36 after exposure to LPS and $PrP_{106-126}$ for 6 and 12 h. We found that both LPS and neurotoxic $PrP_{106-126}$ increased the expression of CD36 at 6th and 12th hour, although CD36 level in $PrP_{106-126}$-treated group was not further increased after 12 h compared with that at 6th hour. Our results were consistent with findings in the literature which supports the assertion of CD36 overexpression in microglia after exposure to amyloid fibril [31,32].

Increased CD36 expression was found in response to $PrP_{106-126}$ stimulation, so we hypothesized that CD36 might be a necessary cell surface receptor in the intracellular signaling pathway leading to iNOS production. We utilized CD36 monoclonal antibody to block CD36 and inhibit the formation of receptor complex. We examined the level of iNOS in the protein extract from BV2 microglia exposed to $PrP_{106-126}$ with or without anti-CD36 antibody. In the present study, results showed that pretreatment with CD36 antibody abolished the $PrP_{106-126}$-induced iNOS up-regulation. These results are consistent with previous studies showing that CD36 mediates free radical production in many neuroinflammatory conditions including Alzheimer disease and cerebral ischemia [14,33,34], and support that CD36 plays a key role in prion diseases-associated oxidative stress by triggering iNOS up-regulation and microglial activation. Taken together, these results establish that microglia employ the cell surface receptor CD36 to detect and respond to insult by PrP peptides.

Although recent findings have provided valuable insights into the role of CD36 in microglial activation induced by $PrP_{106-126}$ *in vitro*, the intracellular signaling molecules responsible for the initiation of these responses remain to be elucidated. Aβ and $PrP_{106-126}$ fibrillar amyloid deposits share an invariant association with reactive glial cells, particularly microglia [22]. Microglia cells respond to fAβ by activating the Src family kinase Fyn as well as the tyrosine kinase Syk, and these kinases have been reported previously to be required for the Src family kinase Fyn as well as the tyrosine kinase Syk in the microglial response to β-amyloid fibrils [21–23]. There is abundant evidence of a microglial-derived inflammatory component in both diseases [22]. We found that PP2 and Pic inhibit $PrP_{106-126}$-induced iNOS production at both mRNA and protein levels in general and that the effect of inhibition at 12th hour was much greater than that at 6th hour at protein level.

PP2 is suggested as a potent and selective inhibitor of Src family kinases, such as $p56^{lck}$ and $p59^{fynT}$ [24]. Fyn is a membrane associated non-receptor protein tyrosine kinase that belongs to the Src family of cytoplasmic tyrosine kinase [35]. It is expressed predominately in tissues of neuronal and hematopoietic origin and has been shown to be involved in CD36-mediated signaling [36]. Kouadir *et al*. [16] in our laboratory has demonstrated that the participation of CD36 in interaction between $PrP_{106-126}$ and microglia may be mediated by Src tyrosine kinases. Our work showed that the Src tyrosine kinase inhibitor, PP2, significantly down-regulated $PrP_{106-126}$-induced iNOS up-regulation in BV2 micrglia, which supported the preliminary results that the participation of CD36 in interaction between $PrP_{106-126}$ and microglia may be mediated by Src tyrosine kinases [16].

Pic is an anti-inflammatory, immunomodulatory, and antiproliferative stilbene that has been shown to interfere with the cytokine signaling pathway [37]. Pic has been shown previously to inhibit the activity of thymocyte p40 kinase [38] and was a potent inhibitor of histamine release in mast cells [39]. Furthermore, *in vitro* assays have showed that the selectivity of Pic for Syk versus Src family kinases holds for Lyn, and Syk is more sensitive than Lyn to the inhibitory activity of Pic [25]. Our work showed that Pic significantly down-regulated $PrP_{106-126}$-induced iNOS up-regulation in BV2 micrglia, suggesting that participation of CD36 in the interaction between $PrP_{106-126}$ and microglia might be mediated by Syk family kinase. Further study is currently underway in our laboratory to prove whether Syk kinases are involved in the pathway of microglia activation.

Evidence in B cells in which membrane immunoglobulin-induced Lyn activation is a rapid and transient process, whereas Syk activation is a slower but longer lasting response, supports this hypothesis [40]. This may give an explanation for our findings that the inhibitory effect of Pic at 12th hour was better than that at 6th hour. On the contrary, the inhibitor effect of PP2 at 6th hour is better than that at 12th hour. Based on our research, the participation of CD36

in the interaction between $PrP_{106-126}$ and microglia might be mediated by multiple signal transductions, which was supported by the result that the decrease of iNOS production in the group of inhibitors PP2 and Pic with $PrP_{106-126}$ or LPS stimulation was more obvious.

ROS, proinflammatory cytokines (e.g. TNF-α, IL-1β, and IL-6), chemokines (e.g. IL-18), and prostaglandins (e.g. PGE2) promote neuronal death [41]. Furthermore, some factors such as TNF-α and IL-1β released by microglia can activate astrocytes, and these factors released from astrocytes may lead to further activation of microglia [42]. qPCR analysis showed that pretreatment of BV2 cells with two inhibitors, Pic and PP2, down-regulated the $PrP_{106-126}$-induced release of IL-1β and TNF-α at 6th and 12th hour, respectively, although the down-regulation was not obvious at 6th hour for TNF-α and at 12th hour for IL-1β (data not shown). The changes of IL-1β and TNF-α mediated by inhibitors were similar to that of iNOS, which suggested that they might share the same signal transduction in microglia exposed to neurotoxic prion peptides.

In conclusion, we have shown that CD36 is involved in $PrP_{106-126}$-induced microglial activation and that PP2 and Pic can abrogate iNOS activation induced by neurotoxic prion peptides in microglia. These findings unveil a previously unrecognized role of PP2 and Pic as Src family kinase Lyn and the tyrosine kinase Syk inhibitor involved in neurotoxic prion peptides–microglia interactions, thus providing new insights into mechanisms underlying the activation of microglia by neurotoxic prion peptides. Although more studies are needed to further explore and confirm these initial findings, our study identified a potential molecular target for the treatment of prion diseases. The detailed knowledge of microglial intracellular signaling pathways may help to find novel therapeutic approaches to prion disease and related diseases.

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