ORIGINAL ARTICLE

GSK3 Inhibition Reduces Inflammatory Responses of Microglia and Upregulates Il-10 Production

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ABSTRACT

Introduction: Neurodegeneration resulting from pathogen invasion or tissue damage has been associated with activation of microglia, and exacerbated by the release of neurotoxic mediators such as pro-inflammatory cytokines, chemokines and reactive oxygen species. Activation of microglia stimulated by lipopolysaccharide is mediated in part by GSK-3 signaling molecule. Induced IL-10 expression via GSK-3 inhibition is noteworthy since IL-10 has been remarkably shown to suppress inflammation. **Objectives:** We aimed to inactivate microglia through inhibition of GSK-3 signaling and to determine its effects on the production of pro- and anti-inflammatory mediators. **Methods:** LPS-stimulated BV-2 cells were treated with a GSK-3 inhibitor (LiCl, NP12, SB216763 or CHIR99021). Inhibition of GSK-3 was determined by the phosphorylation status of GSK-3β. The effects of GSK-3 inhibition on microglial inflammatory response were investigated by examining various mediators and CD200R marker. Production of nitric oxide (NO), glutamate and pro- and anti-inflammatory cytokines were measured using flow cytometry, Griess assay, glutamate assay and Cytometric Bead Array (CBA) respectively. **Results:** GSK-3β signaling in LPS-stimulated microglia was blocked by GSK-3 inhibitor through increased phosphorylation at Serine 9 residue. GSK-3 inhibitors had also led to reducing in microglia activity via increased expression of CD200R. Inhibition of GSK-3 also diminished inflammatory mediators such as nitric oxide (NO), glutamate, pro-inflammatory cytokines (TNF-α and IL-6) and chemokine, MCP-1. Reduction of pro-inflammatory mediators by GSK-3 inhibitor was coincided with increased IL-10 production. **Conclusions:** Suppression of microglia-mediated inflammatory response was facilitated by GSK-3 inhibition with associated increased in IL-10 production.

Keywords: Microglia, BV-2 cell lines, IL-10, GSK-3, Neuroinflammation

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INTRODUCTION

Microglia are the resident immune cells of the CNS, which typically exist in a resting state and regulate various processes (1). Activation of microglia induced by various stimuli, such as lipopolysaccharide (LPS), is commonly accompanied by inflammatory responses to remove damaged cells by phagocytosis (2). Binding of LPS to toll-like receptor 4 (TLR4) on microglia increases expression of MHC class II, CD40, CD80 and CD86 and increases production of pro-inflammatory mediators (3). Chronic microglial activation could confer deleterious effects, which can kill neurons and other neighbouring cells through the release of cytotoxic inflammatory mediators, such as pro-inflammatory cytokines, proteinases and reactive oxygen species (4). Studies have shown that various neuropathological conditions, such as multiple sclerosis, Alzheimer's disease (AD) and Parkinson's disease (PD), are attributed to the activation of microglia (5-7).

Several cell surface markers have been studied to determine microglial activation state including CD200R, an inhibitory marker of macrophages (2). Binding of CD200R to CD200 ligand induces inhibitory signal to the cells, which was found to downregulate inflammation in diseases such as MS (8) and AD (9). The absence of this inhibitory signal, however, has been reported to contribute to the pathogenesis of AD (9) and also worsen the neuroinflammation in PD animal model (10). Upon activation, microglia releases abundant of nitric oxide (NO), which is important as defense molecule against infectious agents and regulate multiple processes of immune cells (11). However, the previous study revealed that high level of NO profoundly inhibits mitochondrial cytochrome oxidase, which leads to neuronal cell death (12, 13). This may be due to competition between NO and oxygen for cytochrome oxidase, which resulted in respiratory inhibition and subsequently neuronal depolarisation

and glutamate secretion (12). In addition, glutamate is the most abundant excitatory neurotransmitter in CNS, plays an important role in learning and memorizing (14). However, overproduction of glutamate may have detrimental effects by causing an imbalance in CNS homeostasis. High level of excitatory glutamate can also cause neuronal cell death by oligodendrocyte killing (15). Impairment of oligodendrocyte will lead to demyelination, the important hallmark of MS (16). Therefore, suppression of microglial activation may prevent the cytotoxic effects of NO and glutamate.

Glycogen synthase kinase (GSK-3) is a protein kinase that plays a role in many cellular processes (17). In the inflammatory processes mediated by microglial activation, GSK-3 has been seen to contribute to inflammation through the production of pro-inflammatory cytokines and increase cell activation and migration (18). Inhibition of GSK-3 through phosphorylation inactivates its protein kinase and negatively regulates the pro-inflammatory responses [19]. Previous findings have shown that inhibition of GSK-3 using short-interference RNA and cells transfection with dominant-negative GSK-3β reduced NO production, but increased expression of IL-10 in both BV-2 cells and primary rat microglia cultures (20).

The chronic inflammatory process commonly contributes to cell damage and tissue injury. However, this process may be retracted by the release of the anti-inflammatory molecule such as IL-10. IL-10 is an anti-inflammatory cytokine produced by various cells in both innate and adaptive immune system, which plays a crucial role in disease protection and prevention of host damage (21). The role of IL-10 has also been discovered as a cytokine that controls pathogenicity of neuroinflammation through inhibition of Th1- and Th17-associated cytokines, particularly IFNγ and IL-17, respectively (22). In addition, the inhibitory action of IL-10 during inflammatory responses could also reduce the activation and differentiation of macrophages, B cells and T cells (23). In relation to GSK-3 signaling, the role of IL-10 has been demonstrated in the LPS-induced endotoxin shock animal model (24). Increased in IL-10 production has been associated with downregulation of pro-inflammatory cytokines through inhibition of GSK-3 (24). These studies have also revealed the crucial role of IL-10 in limiting damage by excessive inflammatory responses. Thus, suppression of GSK-3 activity may be a potential strategy to reduce the pathogenicity of the pro-inflammatory molecules through an increase in IL-10 production. The objectives of this study are to determine the effects of GSK-3 inhibition on the microglial activity, the production of pro-inflammatory mediators and the IL-10 production in the LPS-stimulated BV-2 cells.

MATERIALS AND METHODS

Antibodies and reagents

LPS from Escherichia coli strain O111:B4, Glutamate assay kit and GSK-3 inhibitors such as Lithium Chloride (LiCl), SB216763, NP12 and CHIR99021 were obtained from Sigma-Aldrich (St. Louis, MO). Other reagents include cell culture medium and reagents (Invitrogen, Carlsbad, CA), Griess reagent (Abcam, Cambridge, MA), fluorochrome conjugated anti-mouse CD200R-PE, and Cytometric Bead Array (CBA) inflammation kit (Becton Dickinson Biosciences, San Jose, CA), mouse monoclonal GSK- 3β and phospho-GSK-3β antibodies (Cell Signalling, Danvers, MA) and mouse monoclonal tubulin antibody (Thermo Fisher Scientific, Waltham, MA).

Cell culture

The BV-2 immortalised murine microglial cells was a generous gift from Assoc. Prof. Dr. Thameem Dheen (National University Singapore). Cells were maintained in Dulbecco Modified Eagle Medium (DMEM) (or phenol red-free DMEM for NO production) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 ml/L gentamicin and 250 µg/ml fungizone, 1X non-essential amino acids and 1.5g/l sodium bicarbonate in 95% humidified atmosphere containing 5% CO2 at 37°C. BV-2 cells were stimulated with LPS (1 µg/ml) and treated with either 10 mM LiCl, 10 µM SB216763, 5 µM NP12 or 10 µM CHIR99021 followed by 24 hours incubation.

Griess assay

Cell culture supernatants were collected after 24 hours incubation with LPS and GSK-3 inhibitor (under designated conditions) and combined with the equal volume of freshly prepared Griess reagent (1% sulphanilamide and 0.1% N-1-naphthylenediamine hydrochloride in 2.5% phosphoric acid) in a 96-well plate, followed by 10 minutes incubation at room temperature. The absorbance was measured at 530 nm using microplate reader (Dynex MRX II). Concentration of nitrite was quantitated based on the nitrite standard curve (25).

Glutamate assay

The concentration of glutamate produced by BV-2 cells under designated conditions in supplemented culture medium was measured according to manufacturer's protocol (Sigma). The absorbance was measured at 450 nm using microplate reader (Dynex MRX II) and the concentration of glutamate was calculated based on the generated glutamate standard curve.

Western blot

BV-2 cells were harvested and lysed with sample buffer containing 62.5 mM Tris, 2% SDS, 10% glycerol and 0.0155 g/ml dithiotretol. Electrophoresis was performed on the SDS-PAGE and proteins were transferred to PVDF membrane (Milipore Billerica, MA). The membrane was stained with the primary antibody of total GSK-3β, phospho-GSK-3β (Ser9) or Tubulin at 1:2000 dilution overnight at 4°C, followed by horseradish peroxidase conjugated secondary antibodies (1:10000 dilution) at room temperature for one hour. The bands were visualised using chemiluminescent reagent (Pierce Biotechnology Inc., Rockford, IL), captured using fluorchem imager (Alpha Innotech Corp, San Leandro, CA) and quantified using densitometry with labworks analysis software (UVP).

Expression of CD200R by Flow cytometry

A total of $5x10^5$ BV-2 cells from each designated conditions were stained with 2μg/ml CD200R-PE surface antibody for 30 minutes at 4°C. The cells were acquired on LSRII Fortessa (BD Biosciences, USA) attached with FACSDiva software followed by data analysis using FCAP Array software.

Cytometric Bead Array (CBA)

Cytokine levels produced by BV-2 cells were measured using CBA Mouse Inflammation kit (Cat. No: 552364) according to manufacturer's protocol (BD Biosciences, USA). Cell supernatants were incubated for 2 hours in dark and at room temperature with capture beads conjugated with specific antibody for IL-6, IL-12, MCP-1 and TNF-α. Detection reagent consisting of phycoerythrin (PE) conjugated antibodies were then added into each tube. Beads were acquired using flow cytometer LSRII Fortessa (BD Biosciences, USA) attached with FACSDiva software followed by data analysis using FCAP Array software.

Statistical analysis

Data was presented as mean ± SD with significant changes indicated by * ($p < 0.05$) and ** ($p < 0.01$). Statistical analysis was performed with nonparametric Mann Whitney test and Student's t-test.

RESULTS

LPS increased production of NO in BV-2 cells

Consistent with previous findings, microglial activation induced by LPS can promote NO production (20,26). Our data have shown that a substantial amount of NO was produced from LPS-stimulated microglia in a dose-dependent manner (Figure 1). The moderate concentration of LPS (1 μg/ml) was opted for the subsequent experiment with BV-2 cells due to its ability to induce sufficient amounts of NO (54.85 ± 3.92 μM).

Inhibition of GSK-3 increased phosphorylation of GSK-3β (Ser9) in BV-2 cells

GSK-3 is ubiquitously expressed in many cells including microglia (27). Increased phosphorylation of GSK-3 has been shown to inhibit glycogen synthase activity (28). To determine the role of GSK-3 in microglia, BV-2 cells were cultured with GSK-3 inhibitor in the absence or presence of LPS. The expression of total GSK-3β was remained unchanged in all six different conditions (Figure 2), indicating that the inhibition of GSK-3 did not affect GSK-3β protein level. On the other hand, low expression of phospho-GSK-3β at Serine 9 (Ser9) residue in the presence or absence of LPS alone was observed as compared to those treated with GSK-3 inhibitors, which induced higher levels of phospho-GSK-3β (Ser9) expression. This finding suggests that activation of GSK-3 in microglia was inhibited in the presence of GSK-3 inhibitor through phosphorylation of GSK-3β (Ser9).

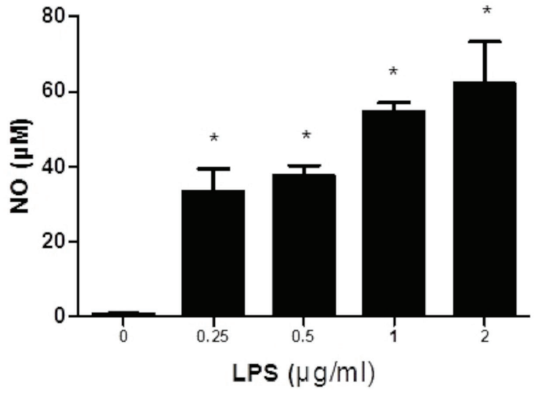

Fig. 1. LPS increased NO production in BV-2 cells.
Cells were cultured in the presence of LPS at various concentrations (0 to 2 μg/ml). Following 24 hours in culture, supernatants were collected and the levels of NO were determined by Griess assay. Data represent the means from three independent experiments performed in triplicates. Values are shown in mean ± SD from three independent experiments; Mann-Whitney test was conducted to compare mean. Statistical significance accepted at p<0.05.

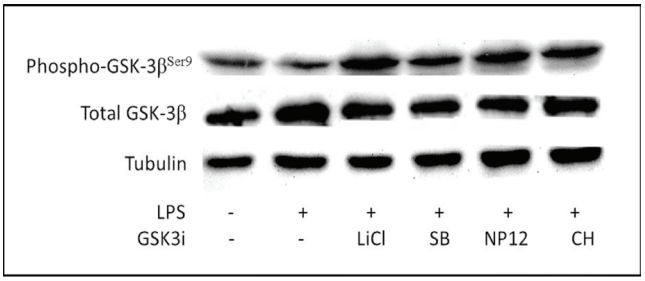

Fig. 2. Inhibition of GSK-3 increased phosphorylation of GSK-3β (Ser9) in BV-2 cells.
Immunoblot analysis of protein levels of total GSK-3β, phospho-GSK-3β (Ser9) and Tubulin in the BV-2 cells that were previously treated with 10 mM LiCl, 10μM SB216763, 5 μM NP12 or 10 μM CHIR99021 for 24 hours in the presence or absence of 1μg/ml LPS. Data shown are representative of three independent experiments.

This inhibitory action of GSK-3 inhibitors may reduce GSK-3β activity without diminishing the amount of protein which has been shown by total GSK-3β.

GSK-3 inhibitor attenuated microglial activation through increased expression of CD200R

Microglial activation status following GSK-3 inhibition was determined by expression of CD200R. Increased expression of CD200R indicates the inhibition of microglial activities by the GSK-3 inhibitor. Results in Figure 3 shows that stimulation of microglia by LPS does not significantly increase median fluorescence index (MFI) of CD200R as compared to unstimulated cells. The MFI of LPS stimulated cells was only 0.1 ± 0.2. However, the presence of GSK-3 inhibitor marginally increased MFI of CD200R. Briefly, LiCl treatment increased MFI up to 81.6 ± 10.12, followed by NP12 (64.3 ± 8.22), CHIR99021 (31.5 ± 6.38) and SB216763 (10.6 ± 4.08). These findings suggest that activation of microglia by LPS stimulation could be revoked by the presence of GSK-3 inhibitors.

Inhibition of GSK-3 reduced NO production in BV-2 cells

GSK-3 inhibitors were able to diminish NO production in microglia that previously stimulated with LPS. Figure 4 demonstrates that low level (0.89 ± 0.22 μM) of NO was produced by microglia during resting state. However, activation of microglia in response to LPS produced substantial amount (54.85 ± 3.92 μM) of NO. Interestingly, treatment of these cells with GSK-3 inhibitors significantly reduced the amount of NO. CHIR99021 has been shown to be the most potent inhibitor in reducing NO levels to 11.41 ± 2.74 μM, followed by LiCl (15.24 ± 1.83 μM), NP12 (20.95 ± 3.72 μM) and SB216763 (27.87 ± 11 μM). These results reveal that inhibition of GSK-3 potentially reduced NO production in activated microglia.

Inhibition of GSK-3 reduced glutamate production in BV-2 cells

Low level of glutamate produced by resting microglia was increased substantially by stimulation of the cells with LPS (43.35 ± 2.38 ng/μl). However, high level of glutamate produced by stimulated microglia was significantly reduced in the presence of GSK-3 inhibitors (Figure 5). LiCl has been shown to be the most potent inhibitor in reducing glutamate production to 15.54 ± 1.33 ng/μl, followed by NP12 (17.07 ± 1.4 ng/μl), CHIR99021 (19.31 ± 1.47 ng/μl) and SB216763 (25.75 ± 1.81 ng/μl). These results suggest that all selective GSK-3 inhibitors potentially reduced glutamate production in the activated microglia.

Inhibition of GSK-3 reduced pro-inflammatory cytokine levels but increased IL-10 production in BV-2 cells

In order to determine cytokine secretion in microglia, the culture supernatant of GSK-3 inhibitor-treated or

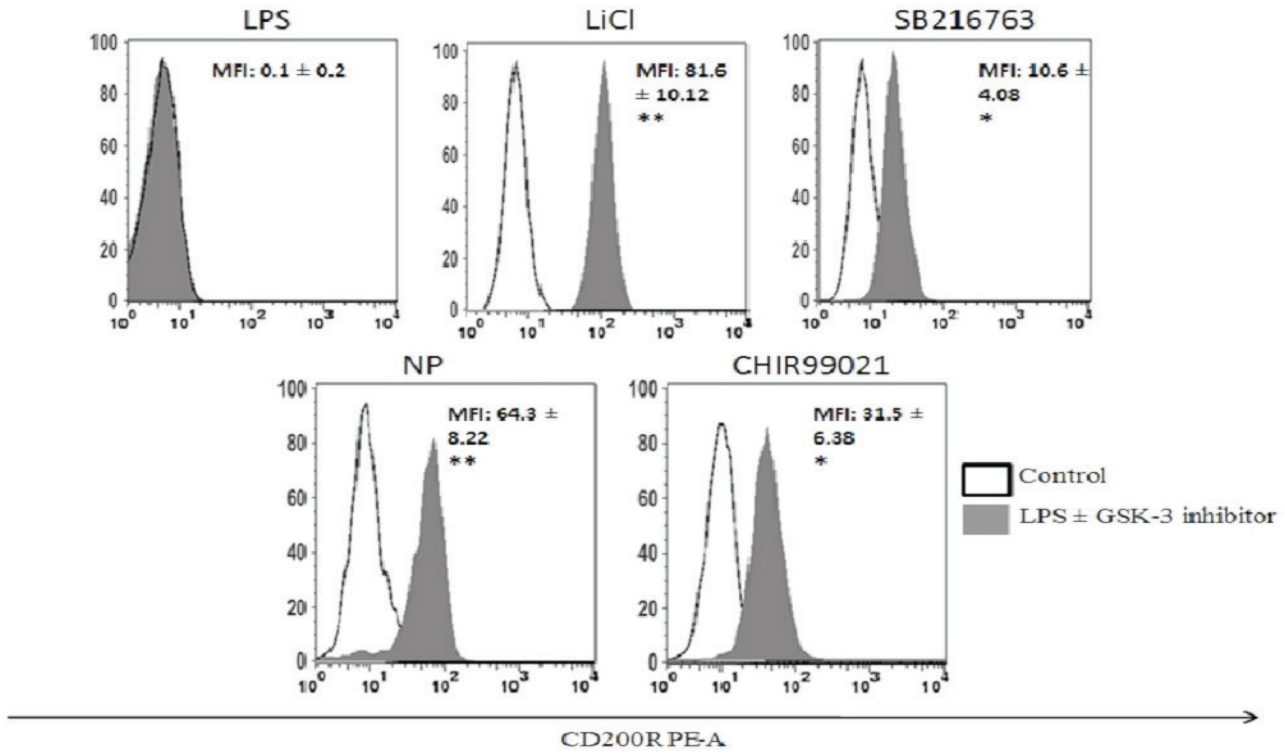

Fig. 3. GSK-3 inhibitor increased expression of CD200R. Histogram overlay showing CD200R-PE Mean Fluorescence Intensity (MFI) of BV-2 cells that were previously treated with 10 mM LiCl, 10μM SB216763, 5 μM NP12 or 10 μM CHIR99021 in the presence or absence of 1μg/ml LPS. Values within histograms correspond to MFI ± SD of the corresponding BV-2 cells, n= 3. Statistical differences are indicated by asterisks **p < 0.01; *p < 0.05; Student's t-test. Results are a representative of three independent experiments

untreated BV-2 cells was collected and the cytokine assay was performed using CBA. Results in Figure 6 show unstimulated BV-2 cells produced negligible amounts of pro-inflammatory mediators. Upon stimulation with LPS, production of pro-inflammatory cytokines including IL-6 and TNF-α, as well as a chemokine, MCP-1, increased to 373.38 ± 74.94 pg/ml, 2817.57 ± 1509.58 pg/ml and 9460.81 ± 2529.46 pg/ml respectively. On the other hand, BV-2 cells treated with LiCl potently reduced IL-6 level to 179.83 ± 33.92 pg/ml and TNF-α level to 1154.31 ± 426.73 pg/ml, whereas NP12 potently reduced the MCP-1 level to 3402.43 ± 1476.84 pg/ml. Interestingly, reduction of all pro-inflammatory mediators by GSK-3 inhibitor has coincided with increased in the production

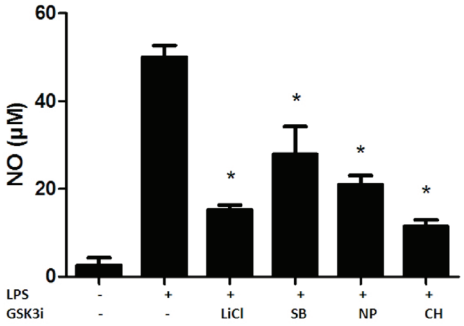

Fig. 4. Inhibition of GSK-3 reduced NO production in BV-2 cells.
BV-2 cell lines (3 × 10^5 cells) were treated with 10 mM LiCl, 10μM SB216763, 5 μM NP12 or 10 μM CHIR99021 in the presence or absence of 1μg/ml LPS for 24 hours. Levels of NO in the cell culture supernatants were determined by Griess assay. Values are shown in mean ± SD from three independent experiments; Mann-Whitney test was conducted to compare mean. Statistical significance accepted at p<0.05.

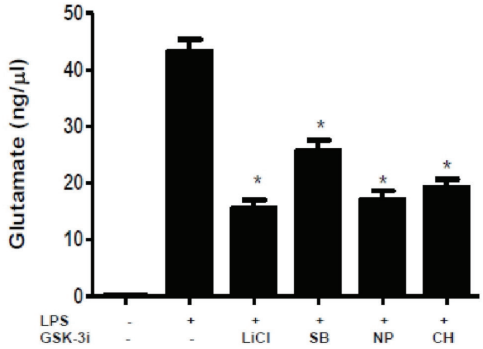

Fig. 5. Inhibition of GSK-3 reduced glutamate production in BV-2 cells.
BV-2 cell lines (3 × 10^5 cells) were treated with 10 mM LiCl, 10μM SB216763, 5 μM NP12 or 10 μM CHIR99021 in the presence or absence of 1μg/ml LPS for 24 hours. Levels of glutamate in te cell culture supernatants were determined by glutamate assay. Values are shown in mean ± SD from three independent experiments; Mann-Whitney test was conducted to compare mean. Statistical significance accepted at p<0.05. Data shown are representative of three independent experiments.

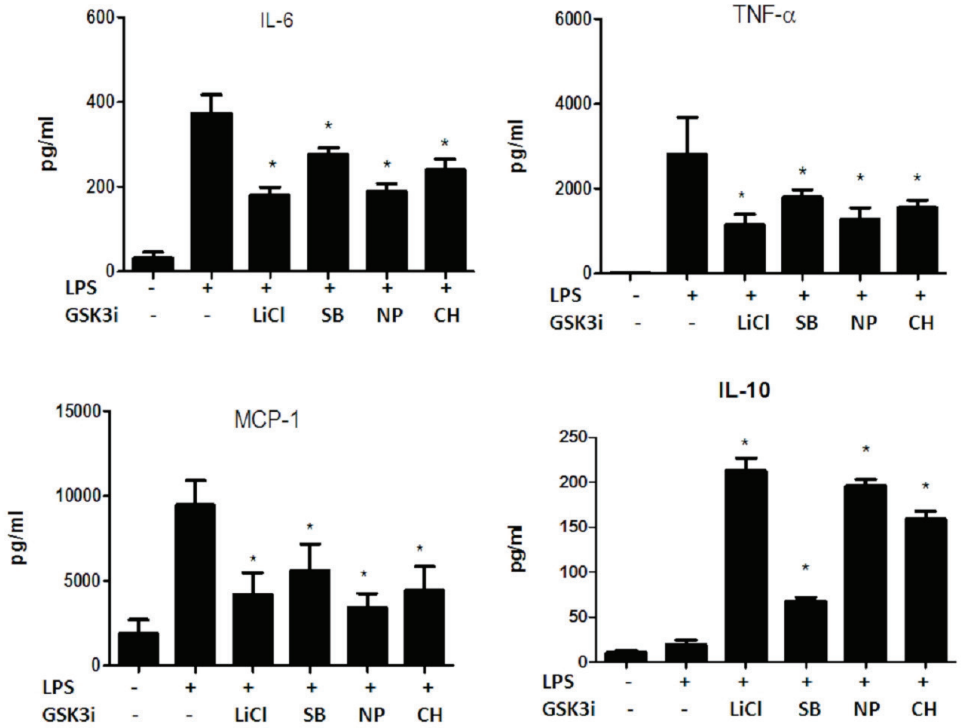

Fig. 6. Inhibition of GSK-3 reduced pro-inflammatory cytokine levels but increased IL-10 production in BV-2 cells. BV-2 cell lines (3 × 105 cells) were treated with 10 mM LiCl, 10μM SB216763, 5 μM NP12 or 10 μM CHIR99021 in the presence or absence of 1μg/ml LPS for 24 hours. Levels of secreted cytokines and chemokine in the cell culture supernatants were determined by CBA. Values are shown in mean ± SD from three independent experiments; Mann-Whitney test was conducted to compare mean. Statistical significance accepted at p<0.05. Data shown are representative of three independent experiments.

of IL-10 up to 212.63 ± 24.08 pg/ml as compared to unstimulated BV-2 cells (10.45 ± 4.12 pg/ml).

DISCUSSION

This study discovers the effects of GSK-3 inhibition on the inflammatory mediators in LPS-stimulated microglial activation. Activation of microglia can be beneficial and crucial in response toward injury that occurs in the CNS parenchyma (29). However, chronic activation of microglia has been considered a hallmark in the pathogenesis of various neurodegenerative diseases by the presence of activated microglia at sites of injury or plaques in the neurodegenerative CNS (30). Various neurotoxins secreted by activated microglia are perceived to contribute to neuronal death and eventual brain injury (30). Therefore, modulating the activation of microglia could be pivotal in limiting inflammation and tissue damage within the CNS. One of the potential strategies to reduce inflammation in microglia is through inhibition of GSK-3. It has been shown that attenuation of microglial activation by GSK-3 inhibitor could limit the cell migration, reduce secretion of inflammatory cytokines and protect the host from inflammation-induced neurotoxicity (18). Similarly, previous findings have also shown that LPS-mediated inflammatory action in microglia can be prevented by the presence of GSK-3 inhibitor (31). Several selective GSK-3 inhibitors (LiCl, NP12, SB216763 or CHIR99021) were chosen in order to block the activation of microglia through inactivation of GSK-3. MTS assay was performed on BV-2 cells with each GSK-3 inhibitors at 10 times higher than the regularly tested concentration to determine the cytotoxicity (data not shown). Interestingly, BV-2 cells remained intact with approximately 95% survival. Among all inhibitors tested in this study, we discovered that LiCl and NP-12 are the most potent inhibitors to inhibit GSK-3 signalling and the efficacy of these inhibitors strongly affirmed in the inhibition of microglial activity, attenuation of pro-inflammatory mediators but, enhanced IL-10 production.

CD200R is a microglial inhibitory marker that has been used to study the microglial activation status. Reduction of CD200R expression indicates activation of microglia. Our data showed that expression of CD200R in microglia had increased following the treatment with GSK-3 inhibitor. It suggests that the inhibitory signal induced by CD200R was enhanced through blocking of GSK-3 signaling in the LPS-mediated microglial activation. We had also found that GSK-3 inhibitors have the ability to inhibit GSK-3 signaling molecule through increased phosphorylation of GSK-3β (Ser9) levels in LPS-stimulated BV-2 cells. Inhibition of GSK-3 through phosphorylation of GSK-3β (Ser9) may exert the protective effect of microglia through blocking of TLR4 expression (24). Previous findings by Martin and colleagues in 2005 have shown that inhibition of TLR4 expression promotes phosphorylation of GSK-3β, which inactivates GSK-3β in microglia (24). These results suggest that inhibition of GSK-3 blocked LPS-induced microglial activation through increased phosphorylation of GSK-3β (Ser9) and expression of CD200R.

One of the potential roles of GSK-3 inhibitor is their ability to downregulate pro-inflammatory components in microglia during inflammation induced by LPS. However, the mechanism of GSK-3-mediating downregulation of inflammatory cytokine is still unclear. Phosphorylation of GSK-3β has been discovered to increase IL-10 production with a significant reduction of pro-inflammatory cytokines (24). Our present data showed that activation of microglia by LPS induced production of several pro-inflammatory mediators, including NO, glutamate, pro-inflammatory cytokines (IL-6 and TNF-α), and the chemokine MCP-1. However, increase phosphorylation of GSK-3β by selective GSK-3 inhibitors was able to diminish the production of all these pro-inflammatory components. Conversely, inhibition of GSK-3 activity had induced the production of IL-10. Similar findings in Huang et al., 2009 have shown that inhibition of GSK-3 in LPS-stimulated microglia decreased the production of NO, but upregulate IL-10 levels (20). However, these studies did not correlate the effects of IL-10 production with other pro-inflammatory cytokines. Interestingly, our data had revealed that reduction of inflammatory components through inhibition of GSK-3 has coincided with the upregulation of IL-10 secretion in BV-2 cells. This finding suggests that IL-10 could play a significant role to dampen down the LPS-mediated pro-inflammatory response in microglia. Moreover, it has been investigated that increased IL-10 levels could limit the immune response to pathogens and prevent damage to the host (32). Thus, reduction of pro-inflammatory mediators through GSK-3 inhibition may be associated with inactivation of microglia and IL-10 production and could be a strategy in amelioration of CNS inflammation.

CONCLUSIONS

Inhibition of GSK-3 may be a crucial strategy to reduce inflammation in the CNS through the production of IL-10. The capability of GSK-3 inhibitor to inactivate microglia and subsequently reduced in pro-inflammatory mediators, demonstrates the possibility of utilising it as a treatment to deal with neuroinflammation. However, the involvement of IL-10 in the downregulation of neuroinflammation remains unclear. Thus, the role of IL-10 in microglia-mediated neuroinflammation could be further determined by the studies of IL-10-knockout mouse model to confirm the effects of GSK-3 inhibition on the pro-inflammatory mediators.

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