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Toll-like receptor 4-mediated signaling participates in apoptosis of hippocampal neurons

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Research Highlights

(1) Toll-like receptor 4 (TLR4) antibody, protein kinase B (AKT) inhibitor, LY 294002, and glycogen synthase kinase 3β (GSK-3β) inhibitor, LiCl, were used in this study to attenuate or augment the effects of the TLR4-phosphatidylinositol 3 kinase (PI3K)/AKT-GSK-3β signaling pathway so as to identify the participation of this signaling system in the apoptosis of hippocampal neurons.
(2) Cell apoptosis is considered the key reason behind the decreased number of neurons in heimer's disease. Thus, a new target for treatment of neurodegenerative diseases would be to block apoptosis signaling pathways in vulnerable neurons.

Abstract

The phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway is considered important for cell survival and has been shown to mediate various anti-apoptotic biological effects. This study explored the role of the Toll-like receptor 4 (TLR4)-mediated PI3K/AKT-glycogen synthase kinase 3β (GSK-3β) signaling pathways in lipopolysaccharide-induced apoptosis in a primary culture of hippocampal neurons. Results demonstrated that the apoptotic ratio of hippocampal neurons stimulated by lipopolysaccharide was significantly higher compared with the control group. Both the expression of P-AKT^{Ser473} and P-GSK-3β^{Ser9} in hippocampal neurons stimulated by lipopolysaccharide decreased compared with the control, while the level of active Caspase-3 and the ratio of Bax/Bcl-2 were significantly increased. The level of active Caspase-3 and the ratio of Bax/Bcl-2 in hippocampal neurons treated with TLR4 antibody or the GSK-3β inhibitor, LiCl, creased before intervention with lipopolysaccharide, but increased after treatment with the AKT hibitor, LY294002. These findings suggest that the TLR4-PI3K/AKT-GSK3β signaling pathway may be involved in lipopolysaccharide-induced apoptosis of hippocampal neurons.

Key Words

neural regeneration; brain injury; hippocampus; neurons; Toll like receptor 4; phosphatidylinositol 3 kinase/protein kinase B-glycogen synthase kinase 3β; apoptosis; grants-supported paper; neuroregeneration

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Conflicts of interest: None declared.

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INTRODUCTION

Neurodegenerative diseases are chronic and progressive and are characterized by neuronal degeneration. The characteristic pathological changes in the majority of neurodegenerative diseases, especially in Alzheimer's disease, are the significantly reduced number of neurons in the brain[1]. The most severely affected regions are the hippocampus and basal ganglia, with neurons decreasing in these regions by an average of 47%. A previous study showed that the massive neurodegeneration seen in patients with early onset familial Alzheimer's disease could be a consequence of an increased vulnerability of neurons with mitochondrial abnormalities, resulting in activation of different apoptotic pathways, indicating that apoptosis may be the major factor for neuronal loss[2]. But, further research is conducted to conclusively understand why neuronal apoptosis occurs in the Alzheimer's brain.

Toll-like receptors (TLRs), members of the pattern recognition receptor family[3-4], are attracting more and more attention from researchers interested in infectious diseases and inflammatory reactions[5] caused by non-pathogenic microorganisms. Microglia and astrocytes can express TLR1-9 in the central nervous system[6-8]; however, only a limited number of TLRs exist on neurons[9-11]. Recently, Czapski *et al* [12-13] found that the mouse hippocampus was affected after an intraperitoneal injection of lipopolysaccharide (1 mg/kg). At 6–24 hours after the systemic administration of lipopolysaccharide, the level of TLR4 rose and corticosterone levels increased, along with inflammation in the blood vessels of the hippocampus. Tang *et al* [14] revealed the expression of TLR2 and TLR4 in cultured cortical neurons, and we have also previously found the expression of TLR4 in hippocampal neurons[15]. What is the potential significance of TLR4 expression in hippocampal neurons? The phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway is considered important for cell survival and can mediate various anti-apoptotic biological effects[16]. Activated AKT mediated a wide range of biological effects by facilitating the phosphorylation of downstream substrates such as glycogen synthase kinase-3β (GSK- 3β)[17-19], the Bcl-2 family and active Caspase-3. Whether the TLR4-mediated PI3K/ AKT-GSK-3β signaling pathway is involved in the process of hippocampal neuronal apoptosis remains controversial.

To confirm that apoptosis of hippocampal neurons is mediated by the TLR4-PI3K/ AKT-GSK-3β signaling pathway, we used LY294002[20-21] to inhibit the PI3K/AKT pathway. LY294002, a widely used specific inhibitor of PI3K/AKT, can decrease the generation of downstream substrate–PIP3 through inhibiting catalysis activity of PI3Kp110 to push the pathway into an inactivated state[22-23]. As a relatively specific inhibitor of GSK-3β[24-25], phosphorylation of serine residues of GSK-3β to produce phosphorylated GSK-3β^{Ser9} (P-GSK-3β^{Ser9}) will inactivate GSK-3β[26]. The content of P-GSK-3β^{Ser9} inversely parallels the activity of GSK-3. Active AKT promotes phosphorylation of GSK-3β, thereby inhibiting apoptosis mediated by GSK-3β. Tang *et al* [14] found that TLR4 could induce apoptosis in a neuronal model *in vitro*, which implied that TLR4 probably participated in PI3K/AKT-GSK-3β pathways of lipopolysaccharide-mediated neuronal apoptosis. As a result, TLR4 might promote ischemic/reperfusion-induced neuronal apoptosis by inhibiting PI3K/AKT-GSK-3β signaling pathways or activating Caspase-3. Nonetheless, the role of TLR4-mediated PI3K/AKT-GSK-3β signaling pathway in lipopolysaccharide-induced apoptosis is still unclear.

In the present paper, we used different compounds to augment or attenuate the effects of the TLR4 signaling pathway at a molecular level. This study aimed to explore the role of TLR4-mediated PI3K/AKT-GSK-3β signaling pathways in lipopolysaccharide-induced apoptosis in primary cultured hippocampal neurons.

RESULTS

Primary culture of hippocampal neurons

After being seeded for 2 hours, cells began to adhere. After 9–14 days, cells clustered and had good refractive power under an inverted microscope. The cell body was stellate and then turned into a round, fusiform or conical shape. Processes were obvious and a dense neural network emerged to cover the culture plate (Figure 1A). Hippocampal neurons were stained red by the specific marker located in the cytoplasm, neuron specific enolase. Nuclei of all cells were stained blue using Hoechst33342. The combination of both markers identified the cells as hippocampal neurons (Figure 1B). Expression of neuron specific enolase-positive cells per field/total cell number in the field of view gives the purity of neurons. The purity in our cultures was more than 95%.

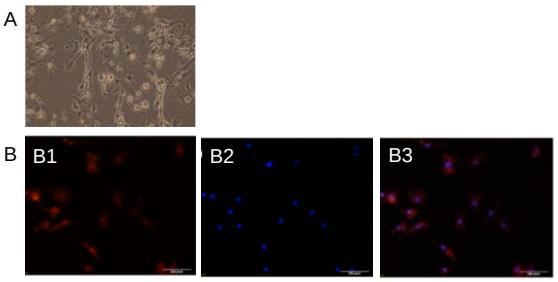

Figure 1 Primary culture and purity of hippocampal neurons (scale bars: 20 μm).

(A) Normal hippocampal neurons after 7 days in culture (inverted microscope).

(B) Identification of hippocampal culture purity (immunofluorescent staining). Hippocampal neurons were labeled with neuron specific enolase (red, B1), and all nuclei were labeled with Hoechst33342 (blue, B2). Merge image: B3.

Stimulation of cell cultures with lipopolysaccharide decreased the viability of hippocampal neurons

The results of CCK-8 indicated that, compared with the control group, cell viability decreased at 0–48 hours after lipopolysaccharide stimulation at both a concentration of 10 and 20 μg/mL. After being stimulated for 24 hours, the activity of cells began to decrease slowly and descended to 69% ($P < 0.01$) and 67% ($P < 0.01$), respectively, compared with the control group (Figure 2A).

The results of flow cytometry indicated that the percentage of apoptosis increased with time. The highest percentage of apoptosis appeared at 48 hours ($P < 0.05$), and the percentage of necrotic cells also peaked at 48 hours (Figure 2B and C).

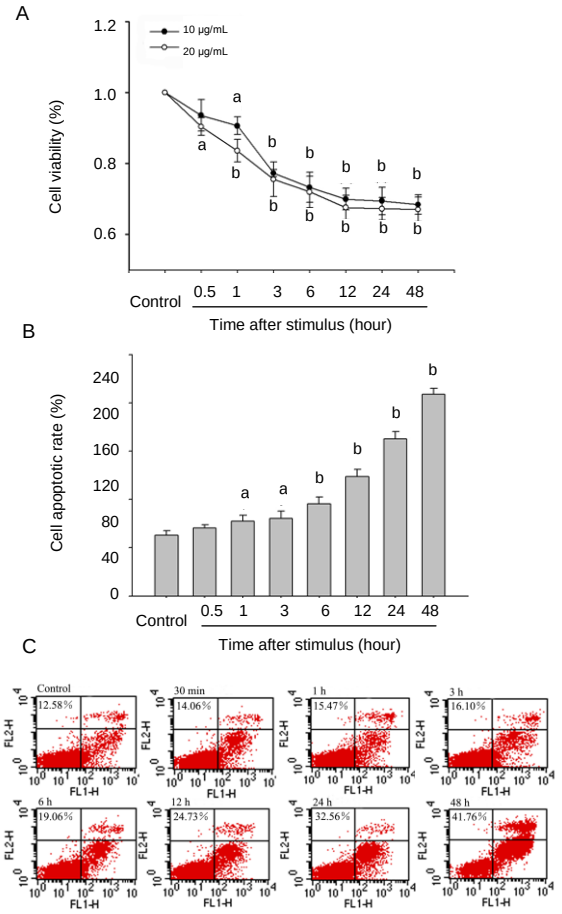

Figure 2 The effect of lipopolysaccharide on hippocampal neurons.

The control group was untreated, while 10 μg/mL and 20 μg/mL lipopolysaccharide were used to treat separate groups of cells.

(A, B) Data are expressed as mean ± SEM. The statistical significance of differences between groups was determined using one-way analysis of variance followed by Tukey's *post hoc* multiple comparison tests. $^{a}P < 0.05$ or $^{b}P < 0.01$, *vs*. control group.

(A) Cell viability of hippocampal neurons at 0–48 hours after lipopolysaccharide stimulation which was detected using CCK-8. Eight wells were detected in each group. (B) Fluoresceine isothiocyanate (FITC)-annexin V/propidium iodide flow cytometry of hippocampal neurons at 0–48 hours after lipopolysaccharide (10 μg/mL) stimulation. Eight wells were detected in each group.

(C) The lower left quadrants of each panel show the viable cells which exclude propidium iodide and were negative for FITC-annexin V binding. The upper right quadrants contained the non-viable, necrotic cells, positive for FITC-annexin V binding and for propidium iodide uptake. The lower right quadrants represent apoptotic cells, FITC-annexin V positive and propidium iodide negative, demonstrating cytoplasmic membrane integrity. One representative experiment out of three is shown.

Inhibition of the PI3K/AKT-GSK-3β signaling pathway affected hippocampal neuronal apoptosis

The results of flow cytometry indicated that the apoptotic

ratio of hippocampal neurons stimulated by lipopolysaccharide was significantly higher than in the control group ($P < 0.05$). The apoptotic ratio of hippocampal neurons treated with TLR4 antibody or GSK-3β inhibitor decreased before the intervention of lipopolysaccharide ($P < 0.05$), but increased after the treatment with LY294002. The exclusive treatment of LY294002 or LiCl had no obvious effect on the apoptotic ratio of hippocampal neurons ($P < 0.05$; Figure 3).

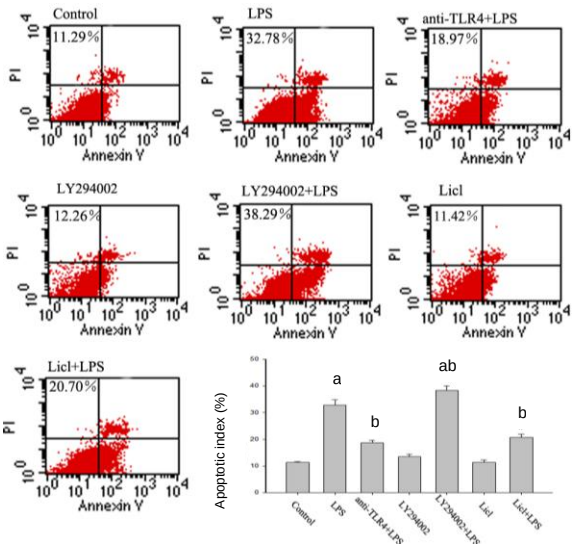

Figure 3 Apoptotic rates of hippocampal neurons following inhibition of the PI3K/AKT-GSK-3β signaling pathway detected using flow cytometry.

Data are expressed as mean ± SEM ($n = 3$). The statistical significance of differences between groups was determined by one-way analysis of variance followed by Tukey's *post hoc* multiple comparison tests. [a]$P < 0.05$ *vs*. control group; [b]$P < 0.05$, *vs*. LPS (10 μg/mL) group. LPS: Lipopolysaccharide (10 μg/mL); anti-TLR4: anti-Toll-like receptor 4.

PI3K: Phosphatidylinositol 3 kinase; AKT: protein kinase B; GSK-3β: glycogen synthase kinase 3β.

Effect of PI3K/AKT-GSK-3β signaling pathway inhibition on the protein expression of AKT, phosphorylated AKT^{Ser473} (P-AKT^{Ser473}), GSK-3β, P-GSK-3β^{Ser9} in hippocampal neurons

Western blot revealed that AKT, P-AKT^{Ser473}, GSK-3β, and P-GSK-3β^{Ser9} were expressed in primary cultured hippocampal neurons. There was no difference in the total expression of AKT and GSK-3β among the groups. The expression of both P-AKT^{Ser473} and P-GSK-3β^{Ser9} in hippocampal neurons stimulated by lipopolysaccharide decreased compared with the control ($P < 0.05$). The effect of lipopolysaccharide was weakened by the treatment of TLR4 antibody before the intervention of lipopolysaccharide ($P < 0.05$). The expression of P-AKT ^{Ser473} further decreased after the treatment with the AKT inhibitor ($P < 0.05$). The expression of P-GSK-3β^{Ser9} in hippocampal neurons increased again after treatment with the GSK-3β inhibitor, LiCl ($P < 0.05$; Figure 4).

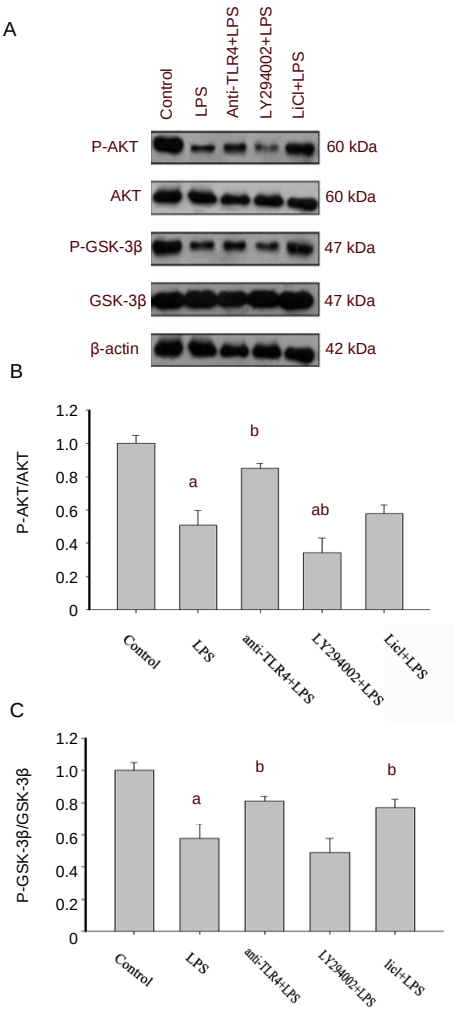

Figure 4 Protein kinase B (AKT) and glycogen synthase kinase-3β (GSK-3β) phosphorylation levels in hippocampal neurons.

(A) Representative western-blots for AKT, phosphorylated AKT^{Ser473} (P-AKT^{Ser473}), GSK-3β and phosphorylated GSK-3β^{Ser9} (P-GSK-3β^{Ser9}) protein from hippocampal neurons.

(B, C) Quantification of P-AKT^{Ser473} and P-GSK-3β^{Ser9} protein levels normalized to the total expression of AKT and GSK3β. The data are expressed as mean ± SEM ($n = 3$). The statistical significance of differences between groups was determined by one-way analysis of variance followed by Tukey's *post hoc* multiple comparison tests. [a]$P < 0.05$, *vs*. control group; [b]$P < 0.05$, *vs*. LPS group.

Inhibition of TLR4 blocked the damage induced by lipopolysaccharide in hippocampal neurons

Immunofluorescence showed that GSK-3β stained green and was located in the cytoplasm in the control group.

After 24 hours of lipopolysaccharide stimulation, the majority of GSK-3β was translocated to the nucleus. However, in the anti-TLR4 + lipopolysaccharide group, the nuclear translocation was obviously reduced (Figure 5).

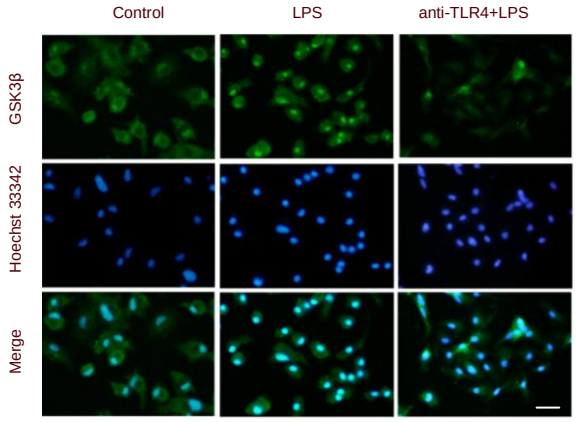

Figure 5 The nuclear translocation of glycogen synthase kinase-3β (GSK-3β) in hippocampal neurons.

The control group was untreated. The lipopolysaccharide (LPS) group was treated with LPS (10 μg/mL). Anti-Toll-like receptor 4 (TLR4) + LPS group was pretreated with TLR4 antibody (10 μg/mL) for 2 hours, and then treated with LPS (10 μg/mL). Hippocampal neurons were stained with Alexa Fluor 488-conjugated anti-GSK-3β antibody (green) and Hoechst 33342 (blue). Scale bar: 10 μm.

Inhibition of the PI3K/AKT-GSK-3β signaling pathway effects apoptosis-related protein expression in hippocampal neurons

We performed western blotting to further examine the expression profiles of Bax, Bcl-2 and active Caspase-3. Western blotting revealed that the level of active Caspase-3 and the ratio of Bax/Bcl-2 in hippocampal neurons stimulated by lipopolysaccharide were significantly increased compared with the control group ($P < 0.05$). The level of active Caspase-3 and the ratio of Bax/Bcl-2 in hippocampal neurons treated with TLR4 antibody or LiCl decreased before intervention with lipopolysaccharide ($P < 0.05$), but increased after the treatment with LY294002 (Figure 6).

Neurodegenerative diseases have very complex etiology and pathogenesis which are not yet fully understood. Study into the mechanisms of neurodegenerative diseases recently extended to the cellular, even molecular level[27]. More and more evidence has been gathered to show that apoptosis is a marker and an ideal target for gene therapy in neurodegenerative diseases. Therefore, exploring the mechanisms of anti-apoptosis will contribute to comprehensive treatment of neurodegenerative diseases and provide new experimental evidence for the further study of specific neuroprotective agents.

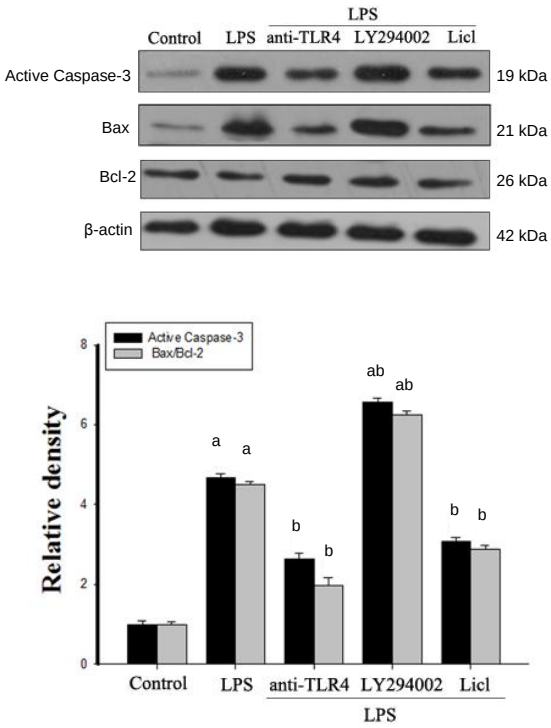

Figure 6 Expression of Bax, Bcl-2, and active Caspase-3 in hippocampal neurons.

(A) Representative western blot of active caspase-3, Bax, and Bcl-2 protein from hippocampal neurons. β-actin was used as a control.

(B) Quantification (relative density) of the intensity of staining of active Caspase-3, Bax, and Bcl-2 protein detected by western blot. Data are expressed as mean ± SEM ($n = 3$). The statistical significance of differences between groups was determined by one-way analysis of variance followed by Tukey's *post-hoc* multiple comparison tests. [a]$P < 0.05$, *vs*. control group; [b]$P < 0.05$, *vs*. LPS group.

DISCUSSION

TLRs are a type of transmembrane pattern recognition receptor, composed of intracellular, membrane and extracellular parts. They are highly conserved from Drosophila to humans and share structural and functional similarities[28]. They recognize pathogen-associated molecular patterns that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity[29]. TLR4 is a member of the TLR family which plays a fundamental role in pathogen recognition and activation of innate immunity. The various TLRs exhibit different patterns of expression. TLR4 has been implicated in signal transduction events induced by lipopolysaccha-

ride found in most gram-negative bacteria. TLR4 has also been shown to be connected with neurodegenerative diseases[30-33].

The PI3K/AKT pathway, composed of PI3K, AKT and their downstream molecules, plays a crucial role in enhancing cell survival[34-35]. AKT, also named protein kinase B, is a kind of serine/threonine protein kinase and can be activated by a variety of nutritional factors to regulate cell resistance to apoptosis. Ser473, located in the regulating regions of the hydrophobic sequence of AKT, is an important phosphorylating site[36-37]. PI3K-activated AKT has multiple downstream target molecules, including GSK-3β, mTOR, the Bcl-2 protein family[38] and Caspase-3[39-40], and can mediate anti-apoptotic biological functions by phosphorylating the above molecules[41]. The activity of GSK3β is inactivated by AKT-induced phosphorylation at ser 9, leading up to anti-apoptosis[42-43]. Phosphorylated GSK-3β is inactive, which is different from other protein kinases. GSK-3β is a ubiquitously expressed kinase that plays a key role in the pathogenesis of neurodegenerative diseases[44-46]. This kinase is capable of acting on some apoptosis-related factors, such as Bax and HSF2[47-49], and is a protein that can facilitate cellular apoptosis. GSK-3β can induce apoptosis by phosphorylating Bax resulting in the entry of Bax into the mitochondria, thereby inducing the release of cytochrome C into the cytoplasm. A large number of studies have confirmed that caspase-3 is located downstream in a series of reactions, and is a key enzyme for the apoptotic process[49], promoting apoptosis through the degradation of corresponding substrates[50-51]. The activation of Caspase-3 depends on the release of mitochondrial cytochrome C, while the release channel of cytochrome C may be regulated by Bax and Bcl-2[52]. When stimulated by the apoptotic signal, Bax, which is normally located in the cytoplasm, transfers to the mitochondrial membrane. The translocation of Bax initiates mitochondria-mediated apoptosis. The anti-apoptotic Bcl-2 protein may play an indirect regulatory role. It has been demonstrated that levels of cell death repressor Bcl-2 and cell death promoter Bax determine the threshold for neuronal cell death, and that their expression is dynamically modulated at the onset of neurodegeneration. Different ratios of Bax/Bcl-2 can affect cellular survival and death.

Rat primary hippocampal neurons were cultured in the present study. According to the results of CCK-8 and flow cytometry, hippocampal neurons stimulated by lipopolysaccharide (10 μg/mL) for 24 hours, were analyzed at a molecular level. The results indicated that lipopolysaccharide can significantly decrease neuronal viability. Quantitative analysis using flow cytometry showed that the amount of apoptosis in the lipopolysaccharide group was significantly increased. Lipopolysaccharide-induced apoptosis can be effectively reduced after anti-TLR4 pretreatment, indicating that TLR4 participated in lipopolysaccharide-induced hippocampal neuronal apoptosis. This study inhibited respective sites of the TLR4-PI3K/AKT-GSK-3β pathway using an antibody against TLR4, LY294002 (a specific inhibitor of PI3K/AKT) and LiCl (a specific inhibitor of GSK-3β), in order to confirm the mechanism underlying hippocampal neuronal apoptosis. We stimulated hippocampal neurons using lipopolysaccharide after the pretreatment of LY294002 and found that the apoptotic ratio was higher than that of the lipopolysaccharide group. After pretreatment of LiCl and subsequent lipopolysaccharide-stimulation, the apoptotic ratio of hippocampal neurons were lower than that of the lipopolysaccharide group. We can detect low AKT activity based on decreased expression of P-AKT^{Ser473} and high GSK-3β activity based on decreased expression of P-GSK-3β^{Ser9} in the lipopolysaccharide group. In the anti-TLR4 + lipopolysaccharide group, the activities of AKT and GSK-3β were opposite to that of the lipopolysaccharide group, and expression of both P-AKT^{Ser473} and P-GSK-3β^{Ser9} was significantly higher than that of the lipopolysaccharide group. The activity of AKT in the LY294002 + lipopolysaccharide group was inhibited due to low expression of P-AKT^{Ser473} (lower than that of lipopolysaccharide group) and the activity of GSK-3β in the LiCl + lipopolysaccharide group was inhibited due to high expression of P-GSK-3β^{Ser9} (higher than that of lipopolysaccharide group). Aoyagi *et al* [53] and Lee *et al* [54] confirmed that the PI3K/AKT pathway was suppressed when neuronal apoptosis occurred in Alzheimer's disease, which was consistent with our conclusions. Immunofluorescence results showed the same trend as the protein results. GSK-3β nuclear translocation was significantly weakened after TLR4 antibody pretreatment. The main change was that GSK-3β transferred from the cytoplasm to the nucleus to exert biological function. Most apoptotic regulatory factors are located in the nucleus; therefore GSK-3β transferred to the nucleus to affect their function, suggesting that the TLR4-PI3K/AKT-GSK-3β signaling pathway was involved in the regulation of lipopolysaccharide-induced neuronal apoptosis.

To further study the role of the TLR4-PI3K/AKT-GSK-3β pathway in mitochondrial apoptosis in hippocampal neurons, we used western blot to detect expression of Bax, Bcl-2 and active Caspase-3 in different groups. The

results from the present study demonstrated that the expression ratio of Bax and Bcl-2 (Bax/Bcl-2) was > 1 after the activation of TLR4, suggesting that factors promoting cellular apoptosis were dominant, thereby further activating active Caspase-3 and triggering the mitochondrial apoptotic pathway. The expression of Bax/Bcl-2, and active Caspase-3 significantly decreased after the TLR4 was blocked. Compared with the lipopolysaccharide group, expressions of Bax/Bcl-2 and active Caspase-3 significantly decreased when AKT was inactivated. Expression of Bax/Bcl-2, and active Caspase-3 significantly increased when GSK-3β was inactivated.

In conclusion, lipopolysaccharide can affect Bax and Bcl-2 protein expression levels by activating TLR4-PI3K/AKT-GSK-3β signaling pathways, thereby activating active Caspase-3 and the mitochondrial apoptotic pathway, ultimately promoting neuronal apoptosis.

MATERIALS AND METHODS

Design

A randomized, controlled, *in vitro*, experimental study.

Time and setting

All the experiments were conducted from December 2010 to May 2012 in the Laboratory of Pathophysiology, Nantong University, China.

Materials

Animals

Twelve 1-day-old male or female specific-pathogen-free Sprague-Dawley rats, were obtained from the Department of Experimental Animal Center of Nantong University, China, with license No. SYXK (Su) 2007-0021. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[55].

Drugs

Lipopolysaccharide (Sigma, St. Louis, MO, USA), polyclonal anti-TLR4 antibody (Santa Cruz Biotechnology, Santa Cruz, USA), LY294002, a specific inhibitor for AKT[20-21], (chemical formula of $C_{19}H_{17}NO_3$; Sigma), and LiCl, a specific inhibitor to GSK-3β[50-51] (Sigma) were used in this study.

Methods

Primary culture of hippocampal neurons

Newborn rats were decapitated, and the skin and skull bone were cut along the center line to harvest the hippocampus[54]. The cleaned hippocampus was transferred to a culture dish with Dulbecco's modified Eagle's medium/F12 (Gibco, New York, USA) and cut into small pieces (approximately 1 mm^3). The tissue suspension was digested with trypsin (final concentration 0.125%; Gibco) for 10 minutes and passed sequentially through 200-mesh Nitex meshes and then centrifuged for 200 × *g*, 5 minutes. The cells were resuspended and seeded at 1–2 × 10^6 cells/mL in 6-well plates with polylysine pretreatment. Cultures were incubated in 5% CO_2 at 37°C and allowed to stand without movement for 12 hours. Twenty-four hours later, medium was replaced with serum-free medium and B27 was added to each well (final concentration 20 μmol/L). Forty-eight hours later, cytarabine (10 μmol/L) was added to each well to inhibit the overgrowth of non-neuronal cells. The medium was then replaced every 3 days.

Experimental groups

The control group remained untreated, while 10 μg/mL and 20 μg/mL lipopolysaccharide groups were treated with these respective concentrations of lipopolysaccharide. Anti-TLR4 (10 μg/mL) + lipopolysaccharide (10 μg/mL) group was pretreated with the anti-TLR4 antibody (10 μg/mL) for 2 hours, and then treated with lipopolysaccharide (10 μg/mL). The LY294002 group was pretreated with LY294002 (50 μmol/L) for 2 hours, whilst the LY294002 + lipopolysaccharide group was pretreated with LY294002 (50 μmol/L）for 2 hours, and then treated with lipopolysaccharide (10 μg/mL). The LiCl group was pretreated with LiCl (10 μmol/L) for 2 hours, and the LiCl + lipopolysaccharide group was pretreated with LiCl (10 μmol/L) for 2 hours, and then treated with lipopolysaccharide (10 μg/mL).

Western blot assay

Western blotting was performed to determine the expression of AKT, P-AKTSer473, GSK-3β, P-GSK-3βSer9, active Caspase-3, Bcl-2 and Bax in hippocampal neurons. Cells (1 × 10^6/mL) were homogenized in radioimmunoprecipitation assay lysis buffer (1% sodium dodecyl sulfate, 1 mmol/L sodium orthovanadate, 10 mmol/L Tris-HCl, pH 7.4) supplemented with a protease inhibitor cocktail. Briefly, samples were loaded and separated on a 12% sodium dodecyl sulfate-poly- acrylamide gel, and proteins were electrotransferred for 2 hours at 4°C onto a polyvinylidene difluoride filter. The membrane was blocked with 5% skim milk powder for 2 hours and incubated with rabbit anti-AKT polyclonal antibody (1:1 000; Cell Signaling, Boston, USA), rabbit anti-P-AKT^{Ser473} polyclonal antibody (1:1 000; Cell Sig-

naling), rabbit anti-rat GSK-3β monoclonal antibody (1:1 000; Cell Signaling), rabbit anti-rat P-GSK-3β^{Ser9} monoclonal antibody (1:1 000; Cell Signaling), rabbit anti-rat active Caspase-3 monoclonal antibody (1:1 000; Bioworld, Minneapolis, MN, USA), rabbit anti-rat Bcl-2 monoclonal antibody (1:1 000; Bioworld), rabbit anti-rat Bax monoclonal antibody (1:1 000; Bioworld), and mouse anti-rat β-actin monoclonal antibody (1:1 000; Santa Cruz Biotechnology) overnight at 4°C. The membrane was then incubated with goat-anti-mouse or goat-anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (1:5 000, Cell Signaling) at 4°C for 2 hours. The immunoreactivity was detected using an Enhanced Chemifluorescence detection system (Millipore, New York, NY, USA)[56].

Immunofluorescent staining

Purified hippocampal neurons were incubated in 24-well plates in which cover slips precoated with polylysine were placed. Subsequently, neurons were fixed in 4% paraformaldehyde diluted with PBS for 30 minutes. Hippocampal neurons were blocked with 10% normal goat serum (diluted in PBS) before exposure to primary antibody. Fixed cells were then incubated with rabbit anti-GSK-3β polyclonal antibody (1:1 000; Cell Signaling) and rabbit anti-neuron-specific enolase polyclonal antibody (1:500; Bioworld) at 4°C overnight. Cells were then incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:1 000, Invitrogen, New York, NY, USA) for 1 hour at 4°C. Nuclei were stained with Hoechst 33342 solution (1:1 000; Dojindo Laboratories, Kumamoto, Japan) for 15 minutes. Samples were observed and photographed with a fluorescence microscope (Olympus, Tokyo, Japan).

Survival rate of hippocampal neurons detected by CCK-8

Cell viability in response to the different treatments was evaluated using CCK-8 (Cell Counting Kit) assay. Cells (1×10^4/mL) were stimulated by 10 and 20 μg/mL of lipopolysaccharide for 0–48 hours. Briefly, after each treatment, CCK-8 reagent (10 μL; Dojindo Laboratories, Tokyo, Japan) was added to each well and the cells were incubated for 2 hours at 37°C and 5% CO_2. The signal was then detected with a microplate reader (SpectraMax 250, Molecular Devices Inc., Sunnyvale, CA, USA) at a wavelength of 450 nm with a background control as the blank. Cell viability was expressed as a percentage of the control cells and was designated as 100%.

Survival rate of cells analyzed by flow cytometric analysis using annexin V and propidium iodide

Cells were seeded in 6-well plates at 2×10^5 cells/well. Cells were washed twice with cold PBS, treated with trypsin and stained with annexin V–fluorescein isothiocyanate (FITC) and propidium iodide in binding buffer (10 mmol/L Hepes, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$). Ten thousand events were collected for each sample. The stained cells were analyzed within 1 hour using flow cytometry (BD Biosciences, San Jose, CA, USA)[57-58].

Statistical analysis

All data were analyzed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). All values are expressed as mean ± SEM. The statistical significance of differences between groups was determined by one-way analysis of variance followed by Tukey's *post hoc* multiple comparison tests. A value of $P < 0.05$ was considered statistically significant. Each experiment consisted of at least three replicates per condition.

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