

Full-length article

Neuroprotective effects of cyclooxygenase-2 inhibitor celecoxib against toxicity of LPS-stimulated macrophages toward motor neuronsYong HUANG, Jing LIU, Li-zhen WANG, Wei-yu ZHANG, Xing-zu ZHU¹*Department of Pharmacology, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Graduate School of Chinese Academy of Sciences, Shanghai 201203, China***Key words**amyotrophic lateral sclerosis; celecoxib; macrophage; NSC34 cell; lipopolysaccharide; cyclooxygenase-2; prostaglandin E₂; nitric oxide; reactive oxygen species¹ Correspondence to Prof Xing-zu ZHU.
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Accepted 2005-05-16

doi: 10.1111/j.1745-7254.2005.00136.x

Abstract

Aim: To establish an *in vitro* injured motor neuronal model and investigate the neuroprotective effects and possible mechanism of celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, on this model. **Methods:** After macrophages were stimulated with lipopolysaccharide (LPS)+interferon- γ (IFN- γ) in the presence or absence of celecoxib for 24 h, the cell-free supernatant of LPS-stimulated macrophages was transferred to the culture of NSC34 cells. Viability of NSC34 cells was assessed by MTT assay after a further 24 h and 72 h incubation. After macrophages were stimulated by LPS+IFN- γ for 12 h or 24 h, the release of prostaglandin E₂ (PGE₂), nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor α (TNF- α) and interleukin-1 β (IL-1 β) from macrophages was measured by radioimmunoassay, Griess assay, fluorescence assay and enzyme-linked immunosorbent assay, respectively. The mRNA levels of COX-2, inducible nitric oxide synthase (iNOS), TNF- α and IL-1 β in macrophages were determined by reverse transcription-polymerase chain reaction after macrophages were stimulated for 6 h and 12 h. **Results:** The supernatant of LPS-stimulated mouse macrophages induced the death of NSC34 cells and celecoxib protected the NSC34 cells against this toxicity. The LPS-induced increases in the release of PGE₂, NO, TNF- α and IL-1 β from macrophages were attenuated by pre-treatment with celecoxib. However, celecoxib showed no effect on the ROS levels upregulated by LPS+IFN- γ in the macrophage supernatant. The mRNA levels of COX-2, iNOS, TNF- α and IL-1 β were increased in LPS-activated macrophages and, except COX-2, reduced by pre-treatment with celecoxib. **Conclusion:** An *in vitro* injured motor neuronal model was established by using the toxicity of LPS-stimulated mouse macrophages toward motor neuronal NSC34 cells. In this model, celecoxib exerted neuroprotective effects on motor neurons via an inhibition of the neurotoxic secretions from activated macrophages.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive fatal neurodegenerative disorder that primarily affects motor neurons in the cortex, brainstem and spinal cord. Evidence suggests that mutations in Cu/Zn superoxide dismutase (SOD-1), glutamate-mediated excitotoxicity, free radical-mediated damage, mitochondrial dysfunction and apoptosis may be involved in the pathogenesis of ALS^[1]. However,

the cause of ALS is not completely understood.

Accumulating evidence indicates that inflammatory processes, especially the activation of microglia, are involved in the pathogenesis of ALS^[2]. Activated microglia are present before the onset of clinical symptoms and prior to significant motor neuron loss in transgenic mice with mutations of the SOD-1 gene, an animal model of ALS^[3]. Furthermore, some critical markers of microglia activation have also been found post-mortem in the cerebral cortex and spinal cord of pa-

tients with ALS^[4,5]. Inflammatory processes would produce harmful effects on neuron survival in ALS tissues, include a prominent upregulation of inducible nitric oxide synthase (iNOS) activity with the subsequent generation of nitric oxide (NO)^[6], the increased generation of reactive oxygen species (ROS) and glutamate^[7], the enhanced secretions of inflammatory cytokines, such as tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6)^[8], as well as the enhanced expression of cyclooxygenase-2 (COX-2) with the subsequent production of prostaglandin E₂ (PGE₂)^[9].

COX-2 was demonstrated to be crucial for prostaglandin synthesis in inflammation. COX-2 expression was shown to be dramatically increased in the spinal cord of both of ALS transgenic mice and ALS patients^[10,11]. PGE₂ levels were also markedly increased in ALS cases compared to non-ALS specimens^[12]. In addition, a selective COX-2 inhibitor, SC236, protected motor neurons in an organotypic cell culture model of ALS^[13]. Furthermore, celecoxib, a highly selective COX-2 inhibitor clinically available for the treatment of rheumatoid arthritis^[14], was proved to prolong survival in a transgenic mouse model of ALS^[15]. These results support a potential role for COX-2 in the neurodegenerative processes of ALS and suggest that a selective COX-2 inhibitor may be effective in the treatment of ALS. However, research on the neuroprotective mechanism of COX-2 inhibition on a cellular level, and drug screens using an injured motor neuronal model are still lacking.

In order to investigate the possible neuroprotective mechanism of the COX-2 inhibitor on ALS and to screen candidate anti-inflammatory drugs for ALS, an injured motor neuronal model, which simulates *in vivo* human microglia activation and the neuronal damage observed during neurodegenerative disease processes, was developed in the present study. Microglia are the resident macrophages of the central nervous system (CNS) as microglia and macrophages, both being cells of the monocyte phagocytic system, have similar biochemical characteristics^[16]. In addition, recent findings suggest that infectious agents may increase the risk of ALS and infected migratory mononuclear phagocytes may play an important role in the infection process^[17]. Therefore mouse peritoneal macrophages as an accessible source of mononuclear phagocytes and neurotoxicity were used. NSC34 cells, a hybrid cell line obtained by fusion of motor neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma N18TG cells, were used as the target motor neuronal cells^[18]. Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, can activate macrophages. Interferon (IFN)- γ is another important stimulant which can enhance cytokine production. A

combination of LPS and IFN- γ was used to activate monocytes^[19,20].

In the present study, we further verified the injured motor neuronal model by evaluating the neuroprotective effect of celecoxib, which prolongs the survival of ALS transgenic mice. In addition, the release of PGE₂, NO, ROS, inflammatory cytokines and the expression of relevant inflammatory genes in macrophages was studied to explore the possible mechanism of the neuroprotective effect of a COX-2 inhibitor against the toxicity of microglia activation on motor neuron viability.

Materials and methods

Drugs and reagents Celecoxib was kindly provided by Dr Yu-she YANG, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. LPS (*Escherichia coli* 055:B5) was purchased from Sigma (St Louis, USA). IFN- γ was obtained from Clonbiotech (Shanghai, China). A radioimmunoassay (RIA) kit for PGE₂ was obtained from China PLA General Hospital (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for TNF- α and IL-1 β were obtained from BD Biosciences (San Diego, CA, USA).

Cell culture Macrophages were obtained from the peritoneal exudates of female BALB/c mice (Grade II, Shanghai Experimental Animal Center, Shanghai, China; Certificate No 003) 4 d after intraperitoneal injection with 0.5 mL 3% thioglycollate (WAKO, Tokyo, Japan). The cells were harvested using cold peritoneal lavage with phosphate-buffered saline (PBS, pH 7.4) containing 1% fetal calf serum (FCS; PAA, Pasching, Austria), washed twice with PBS and resuspended in Dulbecco's modified eagle medium (DMEM)-F12 medium (Gibco, NY, USA) supplemented with 5% FCS, 0.1 g/L streptomycin, and 100 kU/L penicillin. The cells were seeded at a density of 1×10^6 cells/mL at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air in an incubator. After adhering for 2 h, the cells were washed 3 times with warm PBS and incubated in DMEM-F12 medium supplemented with 5% FCS for the formal experiments. The viability of each cell preparation was more than 95%, as assessed by trypan blue staining (0.1% solution).

The mouse motor neuronal NSC-34 cell line was a gift from Dr Jin REN (Shanghai Institute of Materia Medica). NSC34 cells were maintained at 37 °C and 5% CO₂ in DMEM (Gibco) supplemented with 10% FCS, 0.1 g/L streptomycin and 100 kU/L penicillin.

Measurement of cell viability NSC34 cell viability was measured using the MTT assay as described by Hansen *et*

al^[21]. Briefly, MTT (Sigma) was added to cell cultures to reach a final concentration of 0.2 g/L. Following a 4-h incubation at 37 °C, the dark crystals formed were collected and dissolved in 200 μ L/well dimethylsulfoxide in 24-well plates. Subsequently, optical densities were measured at 570 nm by transferring 100 μ L/well to 96-well plates and recording the values using a plate reader (POLARstar[®]; BMG, Victoria, Australia). The number of viable NSC34 cells was calculated as a percentage of the value obtained from the control NSC34 cells incubated with media only.

Measurement of nitric oxide Nitric oxide was determined by assaying for nitrite using Greiss reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 5% H₃PO₄)^[22]. Briefly, 100 μ L of the supernatant from each well (24-well plates) was incubated for 5 min with 100 μ L Griess reagent in 96-well plates. Optical densities of the samples were then obtained by reading absorbance at 540 nm.

Measurement of reactive oxygen species The ROS assay was modified from the fluorescence assay described by Gunasekar *et al*^[23]. Briefly, 90 μ L of supernatant from each well (24-well plates) was transferred to 96-well fluorescence assay plates, incubated to 37 °C, and then added 10 μ L Krab's Ringer buffer (127 mmol/L NaCl, 5.5 mmol/L KCl, 2 mmol/L MgSO₄, 1 mmol/L CaCl₂, 20 mmol/L HEPES, 10 mmol/L dextrose, pH 7.4) with 50 μ mol/L 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma) and 20 IU/mL horseradish peroxidase(SABC, Shanghai, China). The fluorescence value of each well was read at 30 s intervals for 5 min at 485 nm excitation wavelength and 520 nm emission wavelength. The ROS value of each sample was calculated as the slope of its time-fluorescence value curve.

Measurement of PGE₂, TNF- α and IL-1 β The concen-

trations of PGE₂, TNF- α and IL-1 β were determined by RIA and ELISA according to the manufacturer's instructions.

Measurement of mRNA The mRNA levels of COX-2, iNOS, TNF- α and IL-1 β were detected by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using β -actin mRNA as an internal control. Briefly, macrophages from various treatments in 6-well plates were washed twice with ice-cold PBS. Total RNA was extracted from the cells in each well with RNazol (Dingguo Biotechnology, Beijing, China) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out using 2 μ g total RNA and M-MLV reverse transcriptase (Promega, Madison, USA). The reaction mix was incubated at 42 °C for 60 min, and then heated at 70 °C for 10 min. Each reaction mixture was diluted 4 times with 0.1% diethyl pyrocarbonate (DEPC)-treated H₂O. A 2- μ L aliquot from each diluted reaction mixture was used for real-time PCR amplification. The primer sequences and PCR cycle parameters for β -actin^[24], COX-2^[24], iNOS^[25], TNF- α ^[26], and IL-1 β ^[27] are listed in Table 1.

Real-time quantitative RT-PCR was carried out as described by Livak^[28]. Each reaction contained 2 μ L of the cDNA sample with 1 IU *Taq* DNA polymerase (Dingguo Biotechnology) and 0.5 μ L Sybr Green (OPE, Shanghai, China) in a total volume of 20 μ L in a real-time quantitative PCR cycler (DNA Engine Opticon[®] Continuous Fluorescence Detection System; MJ Research, USA). The mRNA level was estimated as a relative value by normalizing with β -actin mRNA. Reaction products were also separated on a 1.5% agarose gel, stained with ethidium bromide, and photographed to validate the reliability of the objective genes.

Statistical analysis Data were presented as mean \pm SD of the values from 3 independent experiments and the

Table 1. Primer sequence and polymerase chain reaction (PCR) cycle parameters for β -actin, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) genes.

| Gene | Primer sequence (sense, antisense) | PCR cycle parameters |
|----------------------------|--|---|
| β -Actin (300 bp) | 5'-GTGGGCCGCTCTAGGCACCAA-3', 5'-CTCTTTGATGTCACGCACGAT TTC-3' | 94 °C 30/45 s, 63 °C 45 s, 72 °C 60/90 s (35 cycles) |
| COX-2 (300 bp) | 5'-TTCAAAGAAGTGCTGGAAAAGGT-3', 5'-GATCATCTCTACCTGAGTGTCTTT-3' | 94 °C 30 s, 63 °C 45 s, 72 °C 60 s (35 cycles) |
| iNOS (947 bp) | 5'-TTTGGAGCAGAAGTGCAAAGTCTC-3', 5'-GATCAGGAGGGATTTCAAAGACCT-3' | 94 °C 45 s, 55 °C 45 s, 72 °C 90 s (35 cycles) |
| TNF- α (200 bp) | 5'-TCTCATCAGTTCTATGGCCC-3', 5'-GGGAGTAGACAAGGTACAAC-3' | 94 °C 45 s, 55 °C 45 s, 72 °C 90 s (35 cycles) |
| IL-1 β (563 bp) | 5'-ATGGCAACTGTTTCCTGAACCTAAC-3', 5'-CAGGACAGGTATAGATTCTTTCCTTT-3' | 94 °C 45 s, 60 °C 45 s, 72 °C 90 s (35 cycles) |

Student's *t*-test was used for the comparison. Values of *P* < 0.05 were considered statistically significant.

Results

Toxicity of LPS+IFN-γ-stimulated macrophages toward NSC34 cells and inhibitory effect of celecoxib on cytotoxicity The supernatant of the macrophages stimulated with a combination of LPS and IFN-γ significantly inhibited the viability of motor neuron NSC34 cells (Figure 1). Adding celecoxib enhanced the survival of these NSC34 cells (Figure 1). The neuroprotective effect of celecoxib was due to an effect on macrophage neurotoxic secretions because celecoxib showed no neuroprotective effect on NSC34 cells if it was added to the supernatant of NSC34 cells directly (data not shown).

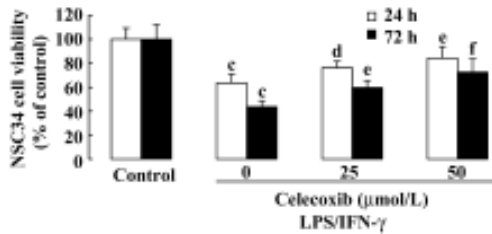


Figure 1. Inhibitory effect of celecoxib on the cytotoxicity of lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated macrophages toward NSC34 cells (MTT assay). Data (mean±SD) from 3 independent experiments (each done in 2 wells, *n*=6) are expressed as a percentage of each control. ^c*P*<0.01 vs control. ^d*P*<0.05, ^e*P*<0.05, ^f*P*<0.01 vs celecoxib 0 μmol/L.

Effect of celecoxib on the production of PGE₂, NO, ROS, TNF-α and IL-1β on LPS+IFN-γ-stimulated macrophages To confirm that the production of PGE₂, NO, ROS, TNF-α and IL-1β was increased in LPS+IFN-γ-stimulated macrophages, their concentrations were measured in the macrophage supernatants. Compared with resting macrophages, LPS+IFN-γ-stimulated macrophages significantly increased the release of PGE₂, NO, ROS, TNF-α, and IL-1β (Figure 2A–2E). To determine whether celecoxib regulates PGE₂, NO, ROS, TNF-α, and IL-1β release from activated macrophages, macrophages were preincubated with celecoxib for 30 min prior to the addition of LPS+ IFN-γ. After the macrophages were stimulated for 12 h or 24 h, the concentrations of PGE₂, NO, ROS, TNF-α, and IL-1β in the macrophage supernatants were measured. The increased productions of PGE₂, NO, TNF-α, and IL-1β were attenuated by preincubation with celecoxib (Figure 2A–2E). However, pre-treatment with celecoxib had no effect on the level of ROS in macrophage supernatants (Figure 2C).

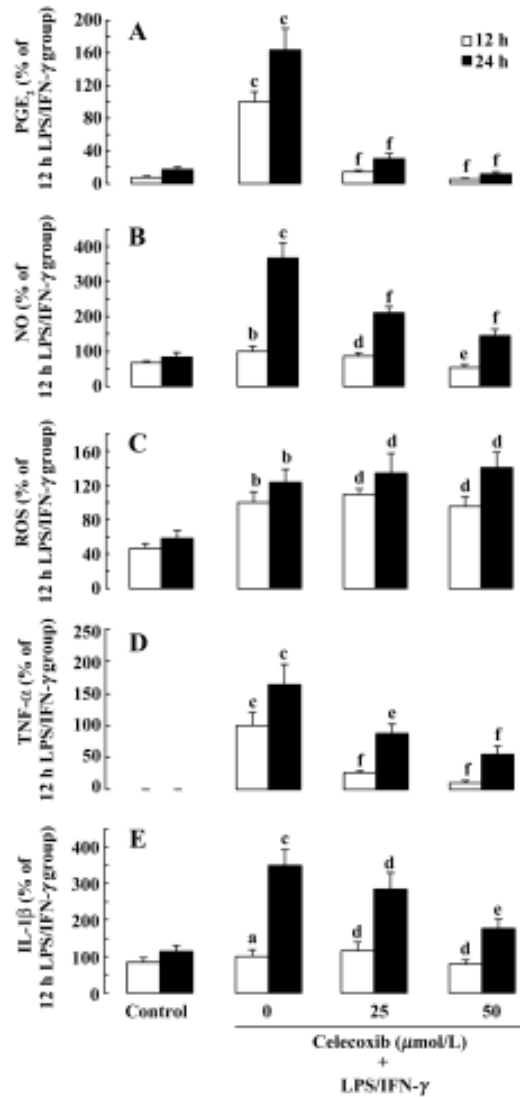


Figure 2. Effect of celecoxib on the release of prostaglandin E₂ (PGE₂), nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated macrophages. Data (mean±SD) from 3 independent experiments (each done in 2 wells, *n*=6) are expressed as a percentage of the values from the 12 h LPS/IFN-γ treatment. ^a*P*>0.05, ^b*P*<0.05, ^c*P*<0.01 vs control. ^d*P*>0.05, ^e*P*<0.05, ^f*P* <0.01 vs celecoxib 0 μmol/L.

Effect of celecoxib on mRNA levels of COX-2, iNOS, TNF-α and IL-1β in LPS+IFN-γ-stimulated macrophages

To determine whether LPS stimulation increases the mRNA levels of COX-2, iNOS, TNF-α, and IL-1β in macrophages, the mRNA levels of these genes were determined by real time RT-PCR. Stimulated macrophages strongly expressed mRNA of COX-2, iNOS, TNF-α and IL-1β following treatment with LPS+IFN-γ (Figure 3A–3D), whereas resting macrophages expressed these mRNA very weakly. Furthermore,

preincubation with celecoxib inhibited the increases of the mRNA levels of iNOS, TNF- α and IL-1 β , which were induced by LPS+IFN- γ in macrophages and had no effect on the mRNA level of COX-2 (Figure 3).

Discussion

In the present study, we demonstrated that the supernatant of macrophages stimulated with LPS 0.5 mg/L plus IFN- γ 1.5×10^5 IU/L caused a loss of NSC34 cells following either a 24-h or 72-h exposure. In consisten with the previous results^[19,20,29], no neurotoxicity was observed when the supernatant from unstimulated macrophages was transferred to NSC34 cells, and LPS (0.0005–5.0 mg/L) increased the neurotoxicity of the supernatant of macrophages on NSC34 cells dose-dependently. LPS did not show toxic effects on NSC34 cells when it was added to the supernatant of NSC34 cells directly (data not shown). These results indicate that NSC34 cells injured by the supernatant of LPS-stimulated macrophages can be used to develop an injured motor neuronal model, whose neurotoxicity was due mainly to inflammatory secretions of macrophages. In the present investigation, Celecoxib was used to verify the model because of its beneficial effects on ALS transgenic mice. It was shown that celecoxib significantly enhanced the survival of NSC34 cells. These results indicate that this model may have valuable applications for drug screening and further research the mechanisms involved. No improvement in NSC34 cell viability was observed when celecoxib was added

directly to NSC34 cells (data not shown). This suggests that the target cells of celecoxib are macrophages rather than motor neurons and that the action of celecoxib on macrophages is likely a reduction of neurotoxic macrophage secretions. Another COX-2 selective inhibitor, NS398, has been reported to have neuroprotective effects on neuronal-like SH-SY5Y cells by suppressing the toxic actions of human monocytic THP-1 cells^[20,29].

PGE₂ is an important mediator involved in a variety of inflammatory processes and COX-2 has been shown to be primarily responsible for the synthesis of PGE₂. COX-2 is rapidly induced by various proinflammatory agents, including LPS, cytokines and mitogens^[30]. In the present study, exposure of LPS to macrophages resulted in an increase in the level of PGE₂ released and an induction of COX-2 expression at the mRNA level (Figure 3). Results from previous studies have also shown that proinflammatory stimuli induce PGE₂ release and COX-2 expression^[30]. Our results showed that PGE₂ release was inhibited by the addition of celecoxib in LPS-stimulated macrophages (Figure 2), suggesting that celecoxib exerts neuroprotective effects on motor neuron NSC34 cells by inhibiting COX-2 activity and the subsequent production of PGE₂ in LPS-stimulated macrophages.

The expression and activity of iNOS plays a pivotal role in sustained and elevated NO release^[31]. It has previously been reported that microglial cells are the main source of LPS-induced iNOS/NO both in neuron-glia culture and *in vivo*^[32]. In addition, there is “cross-talk” between iNOS/NO

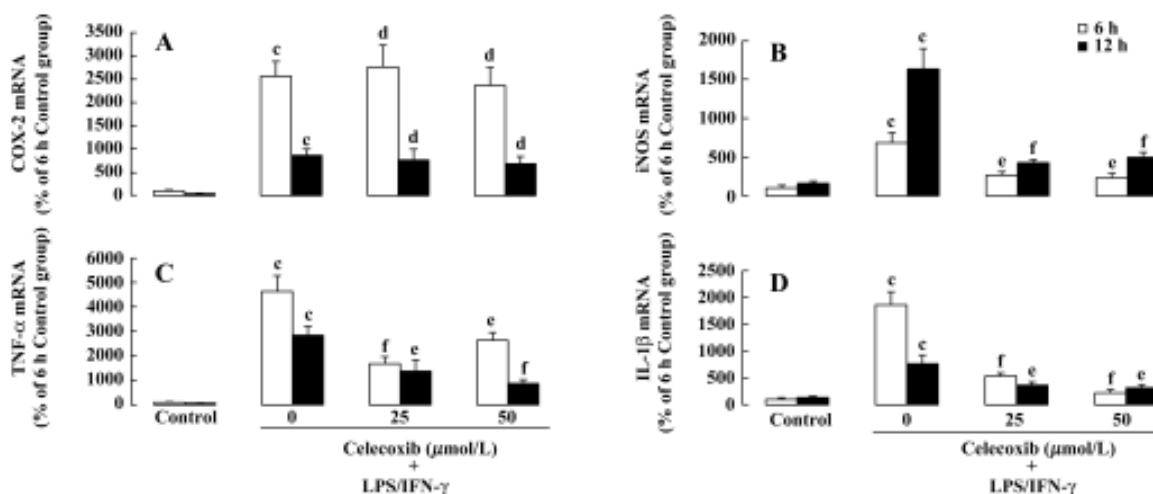


Figure 3. Effect of celecoxib on the mRNA levels of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) in lipopolysaccharide (LPS)/interferon (IFN)- γ -stimulated macrophages. Data (mean \pm SD) are from 3 independent experiments and are expressed as a percentage of the 6 h control. ^c $P < 0.01$ vs control, ^d $P > 0.05$, ^e $P < 0.05$, ^f $P < 0.01$ vs celecoxib 0 $\mu\text{mol/L}$.

and COX-2/PGE₂^[33]. In the present study, we demonstrated that celecoxib significantly inhibits LPS-stimulated iNOS expression and NO release in macrophages. These results suggest that downregulation of iNOS/NO by celecoxib might be involved in the neuroprotective effect of celecoxib against LPS-induced motor neuronal death^[32].

Although one previous study found that LPS-induced ROS was increased in neurons, not microglial cells, in neuron-glia coculture^[33], the most abundant source of oxygen free radicals in the CNS is the respiratory burst system of activated microglia^[2]. A recent study reported that LPS treatment increased intracellular ROS in rat microglia in a dose-dependent manner and ROS played a regulatory role in the expression of COX-2 and the subsequent production of PGE₂ during the process of microglial activation^[34]. On the other hand, Gunasekar *et al* reported that pretreatment with NS398 significantly decreased potassium cyanide (KCN)-induced ROS generation in cerebellar granule cells. The results indicated the involvement of COX-2 in KCN-induced oxidant generation^[23], which further suggests a level of “cross-talk” between ROS and COX-2 in activated microglia. The present studies showed that extracellular ROS levels from LPS-stimulated macrophages were upregulated. However, celecoxib showed no effect on the extracellular ROS level in our study. The results suggest that celecoxib exerts its neuroprotective effect against the toxicity of LPS-stimulated macrophages probably not by the regulation of extracellular ROS.

TNF- α is a potent proinflammatory cytokine that plays an important role in immunity and inflammation. The present study showed that LPS-stimulation increases TNF- α secretion from macrophages. A previous study found that LPS-stimulation increases TNF- α secretion in microglia such as BV-2 cells^[19]. We further demonstrated that celecoxib significantly downregulates TNF- α mRNA level and TNF- α secretion induced by LPS in macrophages. The results suggest that inhibition of TNF- α secretion from the LPS-activated macrophages probably participates in the neuroprotective effect of celecoxib on motor neurons.

Interleukin-1 β is an important cytokine in the inflammation process, and microglia are an important source of IL-1 in the human CNS^[35]. The release of IL-1 plays a critical role in the effect of microglial activation on motor neuron viability and IL-1 is amongst a wide range of factors that upregulate the expression of COX-2 and the subsequent production of proinflammatory cytokines and PGE₂^[9]. Previous studies reported that LPS increases the secretions of IL-1 β in monocytic THP-1 cells^[35]. In addition, another study indicated that celecoxib decreases the secretion of IL-1 β in rats^[36]. The data from the present study show that celecoxib inhibits the

level of IL-1 β , which is increased in LPS-stimulated macrophages. Therefore, the inhibitory effect of celecoxib on the level of IL-1 β may be involved in the mechanism of its neuroprotective effect.

In summary, an injured motor neuronal model was established. The selective COX-2 inhibitor celecoxib showed beneficial effects against motor neuronal death induced by inflammatory reaction. The neuroprotective effect of celecoxib might be associated with downregulation of the levels of PGE₂, NO, TNF- α and IL-1 β as well as gene expression of iNOS, TNF- α and IL-1 β . Since PGE₂^[12], NO^[37-39] and TNF- α ^[19,40] have been reported to be upregulated in ALS, indicating COX-2 inhibitors would be promising candidates for the treatment of ALS.

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