

## Luteolin protects dopaminergic neurons from inflammation-induced injury through inhibition of microglial activation

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### ABSTRACT

Parkinson's disease is a neurodegenerative disorder characterized by progressive degeneration of dopaminergic neurons in the substantia nigra. Accumulating evidence has suggested that inflammation in the brain participates in the pathogenesis of Parkinson's disease. Luteolin, a polyphenolic compound found in foods of plant origin, belongs to the flavone subclass of flavonoids, and has been shown to possess antimutagenic, antitumorogenic, antioxidant and antiinflammatory properties. In this study, we found that luteolin concentration-dependently attenuated the lipopolysaccharide (LPS)-induced decrease in [<sup>3</sup>H]dopamine uptake and loss of tyrosine hydroxylase-immunoreactive neurons in primary mesencephalic neuron–glia cultures. Moreover, luteolin also significantly inhibited LPS-induced activation of microglia and excessive production of tumor necrosis factor- $\alpha$ , nitric oxide and superoxide in mesencephalic neuron–glia cultures and microglia-enriched cultures. Our results demonstrate that luteolin may protect dopaminergic neurons from LPS-induced injury and its efficiency in inhibiting microglia activation may underlie the mechanism.

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Parkinson's disease (PD) is a common neurodegenerative disorder characterized by cardinal features, including resting tremor, slowness of movement, rigidity, and postural instability as a result of progressive and selective degeneration of dopaminergic neurons in substantia nigra (SN) par compacta. The etiology and underlying mechanism responsible for the progressive neurodegeneration of PD remains largely unknown. Increasing evidence suggests that inflammation in the brain, characterized by activation of microglia, might be involved in the pathogenesis of PD [10,13,15]. Under normal conditions, microglia, as the resident macrophages in the brain, serve the role of immune surveillance and host defense [5]. However, in response to injury, infection, or inflammation, microglia become readily activated and secrete a variety of factors, including cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), free radicals nitric oxide and superoxide [10]. Excessive quantities of individual proinflammatory and cytotoxic factors produced by activated microglia are deleterious to neurons. Moreover, these individual factors can work in synergy to aggravate neurodegeneration. The substantia nigra region of the brain is rich in microglia [4].

Over-activation of microglia and excessive release of neurotoxic factors may be a risk factor in triggering the onset of a cascade of events leading to a progressive degeneration of dopaminergic neurons.

Due to the mediating role of microglia, a great deal of effort has been made to develop pharmacological treatment of neurodegenerative diseases including PD by targeting microglia and associative inflammatory factors. In the exploring of more potent neuroprotective agents, many compounds have been found to be effective in inhibition of microglia activation and generation of proinflammatory factors. However, due to unbearable side effects at higher doses, there is no agent which has been established for use in humans. In recent years, there is an increasing interest in focusing on natural products. Some natural products separated from traditional Chinese medicinal herbs have been shown to be neuroprotective *in vitro* and *in vivo*. Wang et al. indicated that andrographolide, a diterpenoid and the major component of *Andrographis paniculata*, reduced inflammation-mediated dopaminergic neurodegeneration in mesencephalic neuron–glia cultures by inhibiting microglial activation [14]. Similarly, Zhou et al. reported that triptolide, an extract of *Tripterygium wilfordii* hook F, protected dopaminergic neurons from inflammation-mediated damage induced by lipopolysaccharide intranigral injection through inhibition of microglial activation [17]. Li et al. also demonstrated that baicalein, a major active component in *Scutellaria baicalensis* Georgi,

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attenuated inflammation-mediated degeneration of dopaminergic neurons by inhibition of microglial activation [7]. Moreover, Tian et al. showed that catalpol, an iridoid glycoside separated from the roots of *Rehmannia glutinosa*, exerted its protective effect on dopaminergic neurons by inhibiting microglial activation and reducing the production of proinflammatory factors [11].

Flavonoids are naturally occurring polyphenolic compounds found in a variety of fruits, vegetables and seeds. Flavonoids have many biological and pharmacological activities including antioxidative, antiinflammatory and antitumor effects. Luteolin, a polyphenolic compound found in foods of plant origin, belongs to the flavone subclass of flavonoids, usually occurring as glycosylated forms in celery, green pepper, perilla leaf and camomile tea. It has been reported to possess antimutagenic, antitumorigenic, antioxidant and antiinflammatory properties. Recent study indicated that luteolin exhibited neuroprotection against oxidative stress-induced cell death in SH-SY5Y neuroblastoma cells [3]. However, very limited investigations have explored the effect of luteolin on the central nervous system. In particular, the effect of luteolin on inflammation-mediated dopaminergic neurodegeneration remains unknown.

In this study, using primary neuron–glia cultures and microglia–enriched cultures, we investigated the effect of luteolin on inflammation-mediated dopaminergic neurodegeneration. We found that luteolin exerted a protective effect against lipopolysaccharide-induced dopaminergic neurodegeneration in mesencephalic neuron–glia cultures and microglia–enriched cultures through inhibition of microglial activation and production of proinflammatory factors.

Luteolin was purchased from Sigma (St. Louis, MO) and the purity was no less than 98%. LPS (*Escherichia coli* O111:B4) and the monoclonal antibody against tyrosine hydroxylase (TH) were purchased from Sigma (St. Louis, MO). [<sup>3</sup>H]dopamine (DA) (30 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA). The monoclonal antibodies against the CR3 complement receptor (OX-42) and glial fibrillary acidic protein (GFAP) were obtained from chemicon (Temecula, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Primary mesencephalic neuron–glia cultures were prepared from the brains of embryonic day 14 Sprague–Dawley rats, following previously described protocol [2,9] with some modifications. Briefly, the ventral mesencephalic tissues were removed and dissociated by a mild mechanical trituration. Dissociated cells were seeded at  $5 \times 10^5$ /well to 24-well culture plates precoated with poly-D-lysine (20  $\mu$ g/ml). The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air in minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 10% heat-inactivated horse serum (HS), 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100  $\mu$ M non-essential amino acids, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin. Seven-day-old cultures were used for treatment. During the course of the experiment, all animals were treated in strict accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals, and all efforts were made to minimize the number of animals and their suffering.

Rat microglia–enriched cultures were prepared from whole brains of one-day-old Sprague–Dawley rats as described previously [9]. The enriched microglia were found to be >98% pure as determined by OX-42- and GFAP-immunoreactive (IR).

Dopaminergic neurons were recognized with anti-TH antibody and microglia were detected with OX-42 antibody, which recognizes the CR3 complement receptor as described previously [2,9]. Briefly, cells were fixed with 3.7% paraformaldehyde in PBS at room temperature for 20 min and then treated with 1% hydrogen peroxide for 10 min. After incubation with blocking solution for 40 min,

the cells were incubated with primary antibodies at appropriate concentrations at 4 °C overnight. Afterward, the cells were incubated with appropriate biotinylated secondary antibody for 2 h followed by the Vectastain ABC reagents for 40 min. Colour was developed with 3,3'-diaminobenzidine. For visual counting of TH-, OX-42-, or GFAP-IR neurons, 10 representative areas per well of the 24-well plates were counted under the microscope at 100 $\times$  magnification. Counting was always performed by two to three individuals in a blind manner.

[<sup>3</sup>H]DA uptake assay was performed as previously described [2,9]. Briefly, cultures were incubated with 1  $\mu$ M [<sup>3</sup>H]DA in Krebs–Ringer buffer (KRB) at 37 °C for 15 min. After washing with ice-cold KRB three times, cells were lysed in 1N NaOH. Radioactivity was determined by a liquid scintillation analyzer. Non-specific DA uptake observed in the presence of mazindol (10  $\mu$ M) was subtracted.

The production of NO was determined by measuring the accumulated level of nitrite (an indicator of NO) in the supernatant using a colorimetric reaction with Griess reagent.

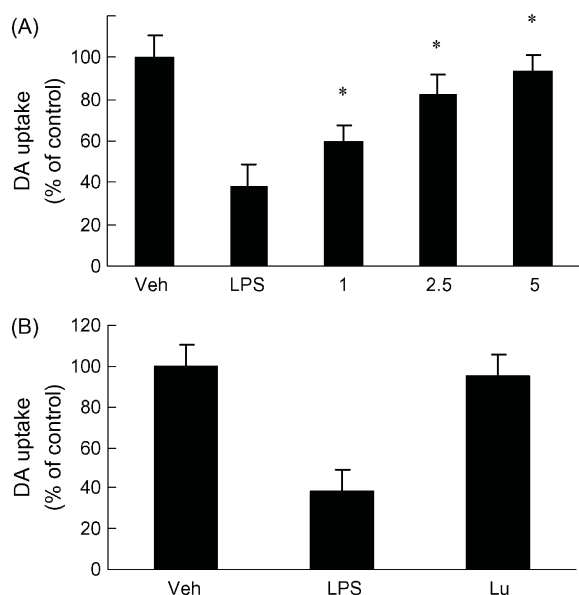
The level of TNF- $\alpha$  in the culture medium was measured with a rat TNF- $\alpha$  enzyme-linked immunosorbent assay kits from R & D Systems (Minneapolis, MN).

The production of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c as previously described [2,9]. Briefly, mesencephalic neuron–glia or microglia–enriched cultures grown in 96-well culture plates were treated with vehicle or LPS in treatment medium containing phenol red-free MEM (150  $\mu$ L/well), with or without 600 U/ml SOD. To each well, 50  $\mu$ L of ferricytochrome c (100  $\mu$ M) in treatment medium was added. The cultures were then incubated at 37 °C for 30 min. Afterward, the absorbance at 550 nm was recorded with a Spectra-Max Plus microplate spectrophotometer. To determine the effect of luteolin on superoxide release, cultures were preincubated with luteolin at 37 °C for 30 min prior to the addition of LPS.

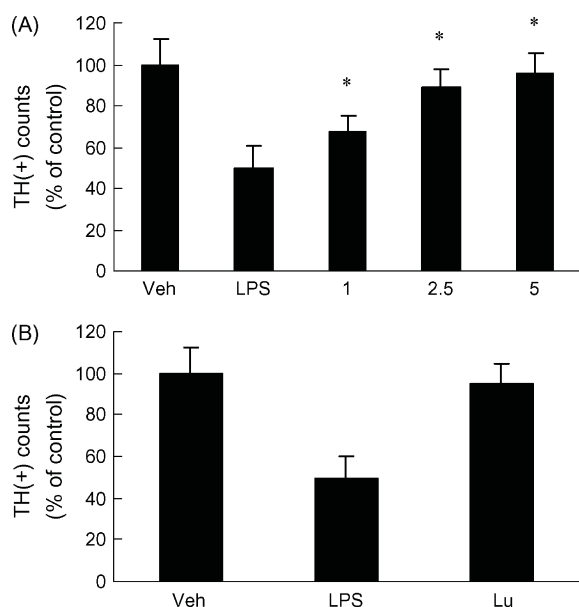
The data were expressed as the mean  $\pm$  SEM. Statistical significance was assessed with one-way analysis of variance (ANOVA) followed by the Newman–Keuls test (SAS software, version 8.0). A value of  $p < 0.05$  was considered to be statistically significant.

Mesencephalic neuron–glia cultures were used to evaluate the effect of different doses of luteolin on LPS-induced dopaminergic neurodegeneration. Mesencephalic neuron–glia cultures were pretreated with vehicle or 1–5  $\mu$ M luteolin for 30 min before the treatment of 10 ng/ml LPS. Seven days later, the degeneration of dopaminergic neurons was assessed by [<sup>3</sup>H]DA uptake or TH immunostaining. As shown in Fig. 1A, [<sup>3</sup>H]DA uptake assays showed that treatment with LPS reduced the uptake capacity by 61.2% compared with vehicle-treated control culture (Fig. 1A). Luteolin significantly attenuated the LPS-induced reduction in DA uptake in a dose-dependent manner. The lowest effective concentration was found at 1  $\mu$ M. DA uptake of cultures pretreated with 1  $\mu$ M, 2.5  $\mu$ M, 5  $\mu$ M luteolin before treatment with LPS were 59.9%, 82.5%, and 93.3% of that of control cultures, respectively (Fig. 1A). But DA uptake of cultures treated with 5  $\mu$ M luteolin alone did not differ significantly from that of control cultures (Fig. 1B).

In addition to DA uptake, counting the number of TH-IR neurons in the cultures revealed that LPS reduced the number of TH-IR neurons by 50.3% compared to vehicle-treated control cultures (Fig. 2A). However, luteolin significantly attenuated LPS-induced reduction in the number of TH-IR neurons in dose-dependent manner, and the number of TH-IR neurons of cultures pretreated with 1  $\mu$ M, 2.5  $\mu$ M, 5  $\mu$ M luteolin before treatment with LPS were 67.5%, 88.4%, and 95.3% of that of control cultures, respectively (Fig. 2A). But treatment of cultures with 5  $\mu$ M luteolin alone did not have significant effect on the number of TH-IR neurons (Fig. 2B).

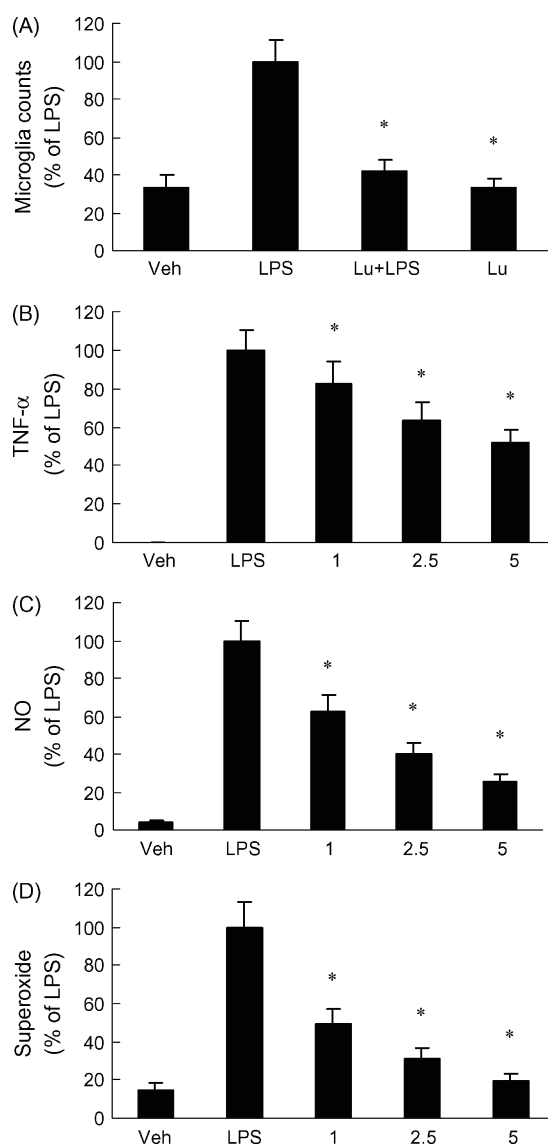


**Fig. 1.** Effect of luteolin on DA uptake in primary mesencephalic neuron-glia cultures. (A) Luteolin (1  $\mu$ M, 2.5  $\mu$ M, and 5  $\mu$ M) attenuated LPS-induced decrease in DA uptake in primary mesencephalic neuron-glia cultures; (B) Luteolin (5  $\mu$ M) alone did not affect DA uptake in primary mesencephalic neuron-glia cultures. Rat ventral mesencephalic neuron-glia cultures were pretreated with vehicle (Veh) or indicated concentrations of luteolin (Lu) for 30 min before treatment with 10 ng/ml LPS for seven days. Results are mean  $\pm$  S.E.M of five experiments performed triplicate and are expressed as a percentage of vehicle-treated control culture. \* $p$  < 0.05 compared with LPS-treated cultures.



**Fig. 2.** Effect of luteolin on the number of DA neurons in primary mesencephalic neuron-glia cultures. (A) Luteolin (1  $\mu$ M, 2.5  $\mu$ M, and 5  $\mu$ M) attenuated LPS-induced decrease in the number of DA neurons in primary mesencephalic neuron-glia cultures; (B) Luteolin (5  $\mu$ M) alone did not affect the number of DA neurons in primary mesencephalic neuron-glia cultures. Rat ventral mesencephalic neuron-glia cultures were pretreated with vehicle (Veh) or indicated concentrations of luteolin (Lu) for 30 min before treatment with 10 ng/ml LPS for seven days. Results are mean  $\pm$  S.E.M of five experiments performed triplicate and are expressed as a percentage of vehicle-treated control culture. \* $p$  < 0.05 compared with LPS-treated cultures.

To elucidate the underlying mechanism of the neuroprotective activity of luteolin, we investigated the effect of luteolin on LPS-induced activation of microglia revealed by OX-42 immunostaining. Culture supernatants were taken at optimal time points for determining the levels of some key proinflammatory factors (TNF- $\alpha$ , NO, and superoxide) and cells were fixed and immunostained for specific markers of microglial activation. As shown in Fig. 3A, after 24 h exposure to 10 ng/ml LPS, the number of OX-42-IR microglia in the cultures significantly increased compared with vehicle-treated control cultures. Interestingly, pretreatment with luteolin (5  $\mu$ M) significantly inhibited LPS-induced increase in the

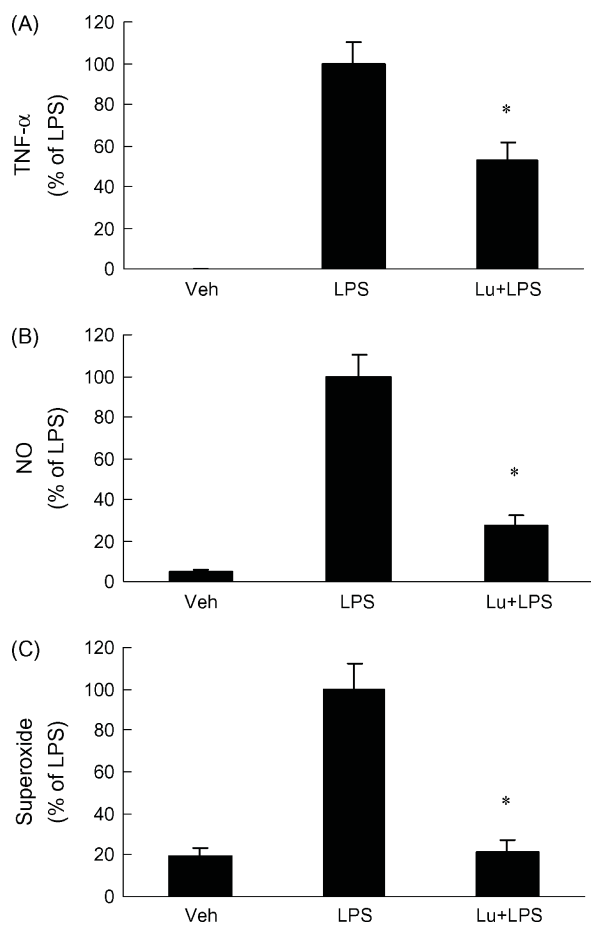


**Fig. 3.** Effect of luteolin on microglia activation and the production of proinflammatory factors in primary mesencephalic neuron-glia cultures. (A) Luteolin (5  $\mu$ M) inhibited LPS-induced microglia activation in primary mesencephalic neuron-glia cultures; (B, C, D) Luteolin (1  $\mu$ M, 2.5  $\mu$ M, and 5  $\mu$ M) inhibited LPS-induced proinflammatory factors (B: TNF- $\alpha$ ; C: NO; D: superoxide) generation in primary mesencephalic neuron-glia cultures. Rat ventral mesencephalic neuron-glia cultures were pretreated with vehicle (Veh) or luteolin (Lu, 5  $\mu$ M) for 30 min before treatment with 10 ng/ml LPS for 24 h. Microglial activation was evaluated by quantification of OX-42-IR microglia. Neuron-glia cultures were pretreated with vehicle or indicated concentrations of luteolin for 30 min before treatment with 10 ng/ml LPS. Supernatants were removed for the measurement of TNF- $\alpha$  at 6 h, and for NO at 24 h. Results are expressed as a percentage of LPS-treated cultures and are mean  $\pm$  S.E.M of five experiments. \* $p$  < 0.05 compared with LPS-treated cultures.

number of microglia, whereas luteolin alone had no effect on their number.

Since luteolin inhibited LPS-induced activation of microglia, we speculated that the production of proinflammatory factors induced by LPS might be decreased by luteolin. To test this hypothesis, we determined the effect of luteolin on LPS-induced production of TNF- $\alpha$ , NO and superoxide in mesencephalic neuron–glia cultures. As shown in Fig. 3B–D, significant levels of TNF- $\alpha$ , NO and superoxide were observed in neuron–glia cultures exposed to LPS treatment. Pretreatment with luteolin inhibited the release of TNF- $\alpha$ , NO and superoxide in a concentration-dependent fashion. Luteolin at 1  $\mu$ M, 2.5  $\mu$ M, and 5  $\mu$ M attenuated the production of TNF- $\alpha$  by 17.4%, 36.9%, and 48.1%, respectively (Fig. 3B). At the equivalent concentration, luteolin reduced the level of NO measured at 24 h after LPS stimulation by 37.1%, 59.7%, and 74.3% (Fig. 3C), and also attenuated the production of superoxide by 50.9%, 69.3%, and 80.6% (Fig. 3D), compared with LPS-treated culture.

In microglia-enriched cultures, we also determined the effect of luteolin on LPS-induced production of TNF- $\alpha$ , NO and superoxide. As shown in Fig. 4A–C, consistent with the results from neuron–glia cultures, pretreatment with luteolin (5  $\mu$ M) significantly inhibited LPS-induced production of TNF- $\alpha$ , NO and superoxide, whereas



**Fig. 4.** Effect of luteolin on the production of proinflammatory factors in primary microglia-enriched cultures. (A, B, C) Luteolin (5  $\mu$ M) inhibited the production of proinflammatory factors (A: TNF- $\alpha$ ; B: NO; C: superoxide) in primary microglia-enriched cultures. Microglia-enriched cultures were pretreated with vehicle or luteolin (Lu, 5  $\mu$ M) for 30 min before treatment with 10 ng/ml LPS. Supernatants were removed for the measurement of TNF- $\alpha$  at 6 h, and for NO at 24 h. Results are expressed as a percentage of LPS-treated cultures and are mean  $\pm$  S.E.M of five experiments. \* $p$  < 0.05 compared with LPS-treated culture.

luteolin (5  $\mu$ M) alone showed no significant effect on the production of these proinflammatory factors (data not shown).

Luteolin, a polyphenolic compound found in foods of plant origin, belongs to the flavone subclass of flavonoids, usually occurring as glycosylated forms in celery, green pepper, perilla leaf and camomile tea. It has been shown to possess antimutagenic, antitumorigenic, antioxidant and antiinflammatory properties. Recent study indicated that luteolin exhibited neuroprotection against oxidative stress-induced cell death in SH-SY5Y neuroblastoma cells [3]. However, to the best of our knowledge, very limited investigations have explored the effect of luteolin on the central nervous system. In particular, the effect of luteolin on inflammation-mediated dopaminergic neurodegeneration has not been reported.

In this study, we use the *in vitro* model of LPS-induced dopaminergic neurodegeneration to investigate the effect of luteolin on dopaminergic neurons. Our results showed that luteolin potentially protected dopaminergic neurons against LPS-induced neurotoxicity by measuring the [ $^3$ H]DA uptake and counting TH-immunoreactive cells. Moreover, luteolin significantly inhibited the activation of microglia and production of proinflammatory factors, including NO, TNF- $\alpha$ , and superoxide, in neuron–glia cultures and in microglia-enriched cultures exposed to LPS treatment.

Microglia, the resident immune cells in the central nervous system, play a role in immune surveillance under normal condition [5]. However, in response to injury, infection, or inflammation, microglia become readily activated and consequently release a variety of factors, including cytokines such as TNF- $\alpha$ , free radicals such as NO and superoxide. These factors are believed to contribute to microglia-mediated neurodegeneration [6]. Many studies indicate that neuroprotection can be obtained by inhibiting microglia activation *in vivo* and *in vitro*. Wang et al. have demonstrated that andrographolide reduces inflammation-mediated dopaminergic neurodegeneration in mesencephalic neuron–glia cultures by inhibiting microglial activation [14]. Similarly, Zhou et al. have shown that triptolide protects dopaminergic neurons from inflammation-mediated damage induced by lipopolysaccharide intranigral injection through inhibition of microglial activation [17]. Moreover, Wang et al. reported that genistein, the primary soybean isoflavone, protected dopaminergic neurons by inhibiting microglial activation [12]. Our previous study also indicated that biochanin A, one of the predominant isoflavones in *Trifolium pratense*, protected dopaminergic neurons against LPS-induced damage through inhibition of microglia activation [1]. These observations suggest that the agents which inhibit microglia activation will provide neuroprotective effects. In agreement with the above reports, in the present study, we first found that luteolin also effectively inhibited microglia activation and the production of TNF- $\alpha$ , NO, and superoxide in mesencephalic neuron–glia cultures and microglia-enriched cultures exposed to LPS treatment. These findings indicated that the mechanism of action underlying the neuroprotective role of luteolin, at least partially, is attributed to the inhibition of microglia activation.

Among a variety of reactive species produced by activated microglia, attention has been paid to reactive nitrogen species due to the prevailing idea that nitric oxide-mediated nitrating stress could be pivotal in the pathogenesis of Parkinson's disease [8]. Excessive accumulation of NO has long been known to be toxic to neurons. The overproduction of free radicals is especially deleterious to neurons. Moreover, when NO meets with superoxide, a more deadly nitrite, peroxynitrite, is formed, which is a potent oxidant and nitrating agent capable of attacking and modifying proteins, lipids and DNA as well as depleting antioxidant defenses. In fact, a recent study has identified peroxynitrite as a key mediator of neurotoxicity induced by LPS-activated microglia [16]. In the present study, we found that LPS induced a robust produc-

tion of both NO and superoxide in mesencephalic neuron–glia cultures and microglia-enriched cultures. However, aside from the inhibition of superoxide, luteolin also significantly inhibited excessive production of NO in mesencephalic neuron–glia cultures and microglia-enriched cultures. Thus, our explanation to the potent neuroprotective effect of luteolin is derived from its dual functions in inhibiting both superoxide and NO production and consequent formation of lethal peroxynitrite.

In this study, we also found that luteolin appeared to be significantly more potent in inhibiting LPS-induced superoxide production than the production of NO and TNF- $\alpha$ . In fact, when mesencephalic neuron–glia cultures stimulated with very low concentrations of LPS (<1 ng/ml), superoxide, other than NO and TNF- $\alpha$ , is the only signal detectable and appear to mediate LPS-induced dopaminergic neurotoxicity [2]. So, we speculate that aside from direct deleterious effect on neuron, superoxide may also play a pivotal role in initiating inflammatory cascade including the production of TNF- $\alpha$  and NO as a “second messenger”. It is possible that agents that have a preferential inhibitory activity toward free radical generation may help to block the deleterious cascade at the very early step, and may subsequently prove to be more effective in providing neuroprotection in the context of inflammation-mediated degeneration. The present study obviously lends support to this notion. However, further investigation in the *in vivo* animal model is required to confirm the effectiveness of luteolin.

In conclusion, our study demonstrated that luteolin protected dopaminergic neurons in the *in vitro* model of LPS-mediated dopaminergic neurodegeneration by inhibition of microglial activation and proinflammatory factors generation. However, the mechanism underlying the neuroprotective effect of luteolin, except inhibition of microglia activation, might not rule out the possibility of other mechanisms. Therefore, further investigation is required to delineate the precise mechanism responsible for neuroprotective effect of luteolin. Therapeutic agents from herbal sources are usually perceived as being natural and devoid of side effects. With the characteristic of high effectiveness and low toxicity, luteolin may be a potential therapeutic agent for the treatment of inflammatory-related neurodegenerative disorders such as Parkinson's disease.

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