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Inhibition of microglial activation by the herbal flavonoid baicalein attenuates inflammation-mediated degeneration of dopaminergic neurons*

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Summary. Accumulating evidence has suggested that inflammation in the brain participates in the pathogenesis of Parkinson's disease (PD). Therefore, anti-inflammatory therapy has attracted much attention as novel interference to neurodegenerative diseases. Baicalein, a major flavonoid extracted from a traditional Chinese herb *Scutellaria baicalensis* Georgi (Huangqin), possesses potent anti-inflammatory and antioxidant properties. To test the potential neuroprotective effect of baicalein on dopaminergic neurons, primary midbrain neuron-glia cultures from E-14 rat embryos were used. Cultures were pretreated with baicalein for 30 min prior to stimulation with lipopolysaccharide (LPS, 10 ng/ml). LPS leads to massive activation of microglial cells revealed by OX-42 immunostaining, and produced excessive quantities of NO. Excessive elevation of superoxide level was also observed in enriched-microglia after stimulating with LPS. LPS-induced damage to dopaminergic neurons was evaluated by uptake capacity for [^{3}H]dopamine and tyrosine hydroxylase (TH)-immunocytochemistry. Pretreatment with baicalein concentration-dependently attenuated LPS-induced decrease in [^{3}H]dopamine uptake and loss of TH-immunoreactive (TH-ir) neurons, which the maximum protective effect was observed at the concentration of 5 μM. Post-treatment with baicalein (5 μM) was also shown to be effective even if baicalein administered up to 2 h later than LPS application. Morphological study shows that baicalein (5 μM) almost completely blocked LPS-induced activation of microglia. Excessive production of TNFα and free radicals such as NO and superoxide by LPS stimulation were also attenuated by baicalein at a concentration-dependent pattern. The present study indicates that baicalein exerts potent neuroprotective effect on LPS-induced injury of dopaminergic neurons. We hypothesize that the inhibition

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of LPS-induced production of NO and free radicals from microglia may underlie the mechanism of baicalein's neuroprotection.

Keywords: Baicalein, neuroprotection, neurodegeneration, inflammation, microglia, Dopaminergic neuron.

Abbreviations

Be baicalein; *DMEM/F12* Dulbecco's modified Eagle's medium/nutrient mixture-F12; *FBS* fetal bovine serum; *LPS* lipopolysaccharide; *MEM* minimum essential medium; *NO* nitric oxide; *PBS* phosphate-buffered saline; *ROS* reactive oxygen species; *TNF*α tumor necrosis factor-alpha

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal features include resting tremor, slowness of movement, rigidity, and postural instability. The major pathological change of PD is the progressive loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Hornykiewicz and Kish, 1987). In contrast to the clear-cut pathological change, the cause and underlying mechanism responsible for the progressive neurodegeneration of PD remains largely unknown (Olanow and Tatton, 1999). There is emerging evidence to support the notion that inflammation in the brain, characterized by the activation of microglia and astroglia, might involve in the pathogenesis of neurodegenerative diseases, including PD, Alzheimer's disease (AD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (Dickson et al., 1993; Liu et al., 2002a; McGeer et al., 1988; McGeer and McGeer, 1995; Giulian, 1999). Under normal conditions, microglial cells, which belong to the mononuclear phagocyte system and are thought as the resident macrophages in the brain, keep in a resting, immunodepressed state and serve the role of immune surveillance (Kreutzberg, 1996). When subjected to abnormal stimulation, such as neurotoxins, neuronal debris, or traumatic brain injury, microglia become gradually activated and produce a host of factors including cytokines, nitric oxide (NO) and reactive oxygen species (ROS) (Liu et al., 2002a). Accumulation of these proinflammatory and cytotoxic factors is deleterious directly to neurons and/or subsequently induces further activation of microglia and finally leads to the progressive degeneration of neurons (Boje and Arora, 1992; Jeohn et al., 1998; McGuire et al., 2001; Chao et al., 1992). Therefore, those compounds possessing anti-inflammatory and/or antioxidant properties have been becoming one of the most promising candidates for PD therapy.

Baicalein (5,6,7-trihydroxy-2-phenyl-4*H*-benzopyran-4-one), which we designate as Be, is one of the major flavonoids of *Scutellaria baicalensis* Georgi, a traditional Chinese herbal medicine (Koda et al., 1970a, b, c). In China, the radix of *Scutellaria baicalensis* has been widely used against various inflammatory diseases, and infections of the respiratory and gastrointestinal tract for centuries (Butenko et al., 1993; Koda et al., 1970c; Gabor, 1986). As the major active component of *S. baicalensis*, baicalein has also been reported to exhibit potent anti-inflammatory effect (Kubo et al., 1984; Sekiya and Okuda, 1982; Wakabayashi,

1999). Baicalein was found to inhibit acetic acid-induced increase in vascular permeability in mice and to reduce acute paw edema in the rats induced by compound 48/80 (Kubo et al., 1984). It also suppressed development of secondary lesion in adjuvant-induced arthritis in rats (Kubo et al., 1984). Antioxidant effect of baicalein is also well documented (Hanasaki et al., 1994; Yoshino and Murakami, 1998; Gao et al., 1999). In this regard, it has been reported to scavenge reactive oxygen species (ROS), including superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals (Hamada et al., 1993). Therefore, we speculate that baicalein, with the anti-inflammatory and antioxidant properties, may exert the capacity to block the deleterious events of endotoxin lipopolysaccharide (LPS)-activated microglia and consequently protect inflammation-mediated degeneration of dopaminergic neurons. To achieve this goal, we use the in vitro model of LPS-induced dopaminergic degeneration to investigate the protective effect of baicalein. This model has been proved to successfully mimic most of the phenomena and process of inflammation-mediated dopaminergic degeneration (Liu and Hong, 2003).

Methods

Reagents

Baicalein (98% in purity) was purchased from Sigma-Aldrich (St. Louis, MO). Dihydroethidium was purchased from Molecular Probe (Eugene, OR). Dihydroethidium was prepared as 10 mM stock in dimethylsulfoxide and stored at -80°C until use. Working stocks (2 μM) were made in distilled water and the final DMSO concentration in the cell culture was less than 0.1%. Cell culture ingredients were obtained from Invitrogen (Carlsbad, CA) and [^{3}H]DA (30 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA). The monoclonal antibody against the CR3 complement receptor (OX-42) was obtained from Pharmingen (San Diego, CA). The polyclonal anti-tyrosine hydroxylase (TH) antibody was a gift from Dr. John Reiherd of GlaxoSmithKline (Research Triangle Park, NC). A Vectastain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA).

Preparation of rat mesencephalic neuron-glia cultures and treatment with test medicine

Primary mesencephalic neuron-glia cultures were prepared from the brains of embryonic day 14/15 Fischer 344 rats, following our protocol previously described (Liu et al., 2000). Briefly, the ventral mesencephalic tissues were removed and dissociated by a mild mechanical triturating. Cells were seeded at 5×10^5/well to 24-well culture plates pre-coated with poly-D-lysine (20 μg/ml) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in 0.5 ml/well maintenance medium. The medium consists of 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 μM non-essential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin. Three days after the initial seeding, 0.5 ml of fresh medium was added to each well. Seven-day-old cultures were used for experiment. The composition of the cultures at the time of treatment was approximately 48% astrocytes, 11% microglia, 40% neurons and ~1% TH-ir neurons.

To investigate its protective potential, the cultures were pretreated with baicalein (1 ~ 5 μM) made in treatment medium (MEM with 1% FBS, the other components are same as maintenance medium) 30 min prior to LPS (10 ng/ml) application and was kept in the medium throughout the experiment.

A post-treatment scheme was designed to examine the potential therapeutic effect of baicalein. In this scheme, LPS (10 ng/ml) was added first and subsequently baicalein (5 μM) was applied at indicated time points (0 ~ 120 min). Fifty micro liters of supernatant per well was

collected for Griess nitrite assay 48 h after LPS treatment. Both [^{3}H]DA uptake assay and immunocytochemistry were conducted 7 d later.

[^{3}H]DA uptake assay

[^{3}H]DA uptake assays were performed as previously described (Liu et al., 2000). Briefly, Cultures were incubated for 20 min at 37°C with 1 μM [^{3}H]DA in Krebs-Ringer buffer (KRB, 16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). After washing three times with ice-cold KRB, cells were lysed in 1 N NaOH. Radioactivity was determined by Packard liquid scintillation analyzer (TRI-CARB 2900TR, Packard BioScience Co., Meriden, CT). Non-specific DA uptake observed in the presence of mazindol (10 μM) was subtracted.

Immunocytochemistry

Dopaminergic neurons were recognized with anti-TH antibody and microglia was detected with the OX-42 antibody, which recognizes the CR3 receptor as previously described (Liu et al., 2000). Briefly, formaldehyde (3.7%)-fixed cultures were treated with 1% hydrogen peroxide (10 min) followed by sequential incubation with blocking solution for 30 min, primary antibody (overnight, 4°C), biotinylated secondary antibody (2 h), and ABC reagents (40 min). Color was developed with 3,3′-diaminobenzidine (DAB). For morphological analysis, the images were recorded with a Nikon inverted microscope connected to a charge-coupled device camera (DAGE-MTI, Michigan City, IN) operated with MetaMorph software (Universal Image Co., West Chester, PA). For visual counting of TH-ir neurons, nine representative areas per well of the 24-well plate were counted under the microscope at 100× magnification. To measure the average TH-ir dendrite, 50 TH-ir neurons in each well were randomly chosen and 3 wells for each treatment condition were used.

mRNA expression evaluated by RT-PCR

To examine the effect of baicalein on LPS-induced expression of iNOS mRNA, BV2, a murine microglial cell line, was used. Certain concentrations of baicalein as described in results was added 30 min prior to LPS (10 ng/ml) application. 3 h later, media was removed and TRI reagent from Sigma was used for the extraction of total RNA from cells. Yield and purity of RNA preparations were checked spectrophotometrically at 260 and 280 nm. One μg of total RNA from each sample was used for cDNA synthesis. Reverse transcriptions were performed using the superscript^{TM} first-strand synthesis system for RT-PCR (Invitrogen) following the manufacturer's directions. PCR was performed using Taq DNA Polymerase from Invitrogen. The following primers derived from the published cDNA sequences were used for the PCR amplifications: iNOS forward 5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′; iNOS reverse 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′; GAPDH forward 5′-CAT TGA CCT CAA CTA CAT GGT-3′; GAPDH reverse 5′-TTG TCA TAC CAG GAA ATG AGC-3′.

PCR was performed at 94°C for 1 min, at 64°C for 1 min and at 72°C for 1 min. To ensure that the amplification was still in the exponential range, reactions were stopped at specific cycle numbers for the different primer pairs: for iNOS, 26 cycles; for GAPDH, 23 cycles. Products were inspected visually on 1.5% precast agarose gel with ethidium bromide staining (Invitrogen, San Diego, CA). Bands were quantified by densitometry. Counts for background were subtracted from counts for the specific bands for the iNOS or GAPDH signals. Ratios were calculated for iNOS signals with the control signals from GAPDH. Averages from these ratios were presented.

Measurement of nitrite concentration

The production of NO was determined by measuring the accumulated levels of nitrite in the supernatants by a microplate assay method, as previously described (Liu et al., 2000). To measure nitrite, 50 μl aliquots were removed from supernatants of cultured cells and incubated with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm

was determined in a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentration of nitrite was calculated using sodium nitrite as a standard.

Superoxide assay

Detection of intracellular superoxide production was done in BV2, a murine microglial cell line, by a dihydroethidium staining method. Dihydroethidium can be oxidized by superoxide to fluorescent ethidium, which is retained intracellularly by stably binding to DNA and RNA. Briefly, BV-2 cells (5×10^4) were cultured on coverslips. After overnight incubation, individual cell cultures were treated with various reagents for 2.5 hours. The cultures were treated with/without baicalein 30 min prior to addition of LPS and then stained with dihydroethidium (2 μM) for 20 min following by fixing with 10% buffered formalin. The production of superoxide was observed by the appearance of red fluorescence using LSM 510 confocal microscope at excitation wavelength of 488 nm, dichroic mirror of 505 nm and emission >510 nm. Images were digitialized as 256×256 pixels.

Statistical analysis

The data were expressed as the mean ± SEM. Statistical significance was assessed with an analysis of variance (ANOVA) followed by Newman Keul's post-hoc test using the GraphPad Prizm 3.0 (GraphPad Software, Inc., San Diego, CA). A value of $p < 0.05$ was considered to be statistically significant.

Results

Effect of baicalein on LPS-induced degeneration of dopaminergic neurons

Mesencephalic neuron-glial cultures were pretreated for 30 min with vehicle or 1–5 μM baicalein prior to application of 10 ng/ml LPS. Seven days later, the

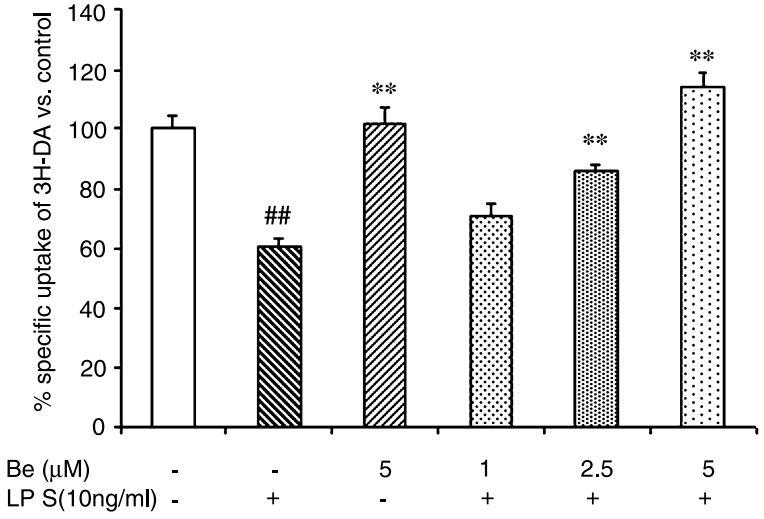

Fig. 1. Effect of baicalein (Be) on LPS-induced degeneration of dopaminergic neurons on mesencephalic neuron-glia cultures. Cultures were treated with vehicle, or indicated concentrations of baicalein 30 min prior to treat with 10 ng/ml LPS. Seven days later, neurotoxicity was assessed by [^{3}H]-labelled DA uptake as described in Methods. Data are expressed as percentage of the control group and presented as mean ± SEM of 3 experiments performed in triplicate. $^{\#\#}p < 0.01$ compared with vehicle control and $^{**}p < 0.01$ compared with LPS-treated group

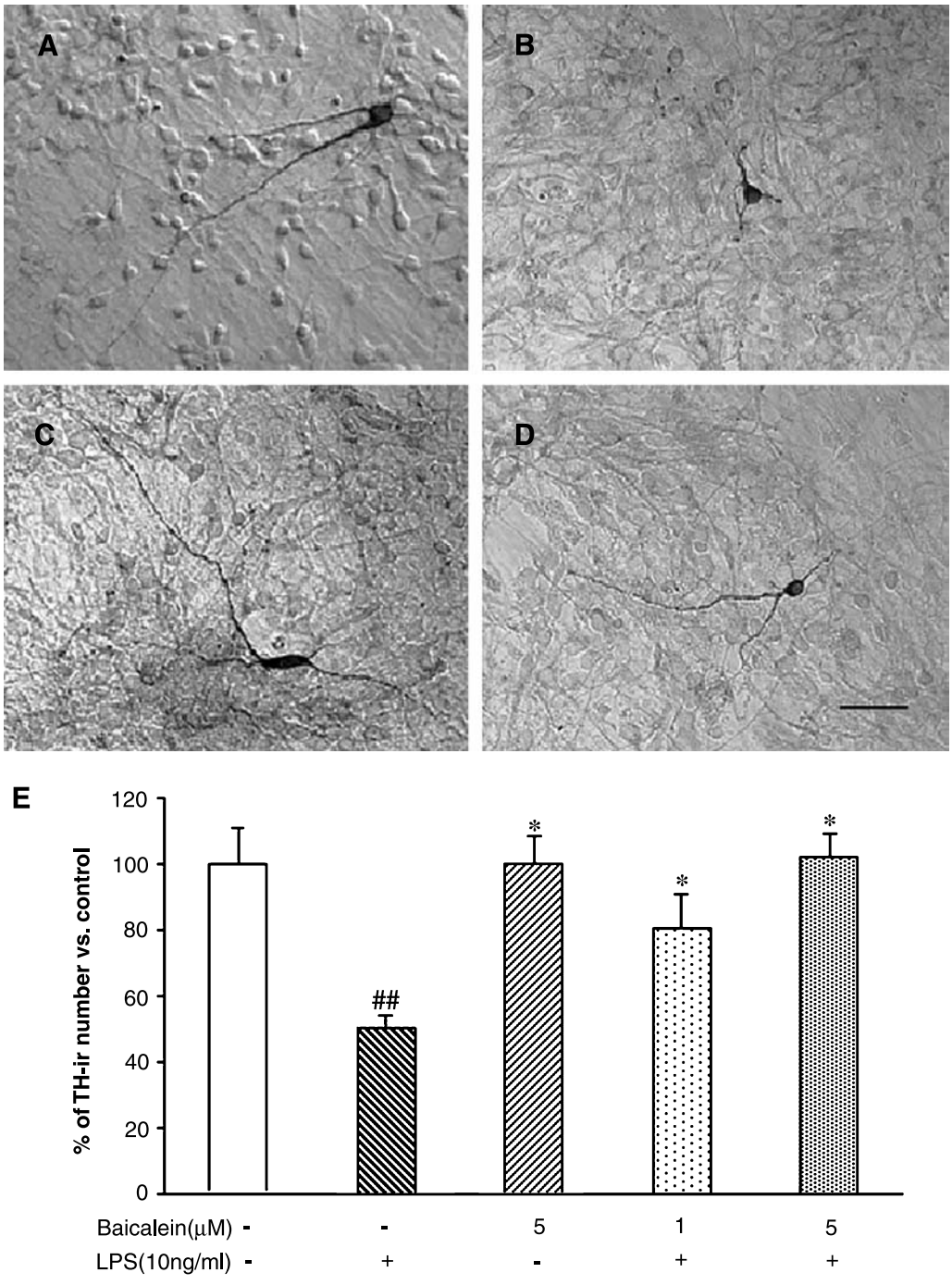

Fig. 2. Morphological evidence of the protective effect of baicalein (Be) against LPS-induced damage to dopaminergic neurons in mesencephalic neuron-glial cultures. After maintaining in maintenance medium for seven days, cultures were changed with treatment medium and treated with vehicle or different concentrations of baicalein 30 min prior to treat with 10 ng/ml LPS. TH immunocytochemistry was performed to reveal dopaminergic neurons after treated for seven day. Images were captured with SPOT-2 cooled CCD combined with MetaMorph software. **A** control group (vehicle alone); **B** LPS (10 ng/ml) alone; **C** Be (5 μM) alone; **D** Be (5 μM) with LPS. The magnification is 200× and bar = 250 μm. The total number of TH-ir neurons in each well was counted under converted microscope and data were presented as mean ± SEM (n = 3).

$^{\#\#}p < 0.01$ compared with vehicle control; $^{*}p < 0.05$ compared with LPS-treated group

degeneration of dopaminergic neurons was assessed by [^{3}H]DA uptake or TH immunostaining. [^{3}H]DA uptake assays indicated that treatment with LPS reduced the uptake capacity by about 40% *versus* control group (Fig. 1). Baicalein significantly attenuated the LPS-induced decrease in DA uptake, in a dose-dependent manner. The most effective concentration was found at 5 μM, which completely reversed LPS-mediated toxicity. DA uptake of cultures treated with 5 μM baicalein alone did not differ significantly from that of control cultures, means devoid of obvious toxicity (Fig. 1).

Counting the number of TH-ir neurons in the cultures revealed that LPS reduced the number of TH-ir neurons by 49% compared to vehicle-treated control cultures (Fig. 2E). Baicalein (5 μM) significantly attenuated LPS-induced reduction in the number of TH-ir neurons (Fig. 2E). Morphologically, in addition to the reduction in abundance, the remaining TH-ir neurons in LPS-treated cultures had a significantly fewer dendrites, shorter or even truncated axons (Fig. 2B). In cultures pretreated with baicalein (5 μM), the number of TH-ir neurons was significantly more numerous and the TH-ir dendrites were less affected compared with that of LPS-treated cultures (Fig. 2D). Measurement of the average length of TH-ir dendrites indicated that the average dendrite length of LPS-treated cultures was 5.2% of control cultures and that of the cultures pretreated with baicalein prior to LPS stimulation was 81% of control.

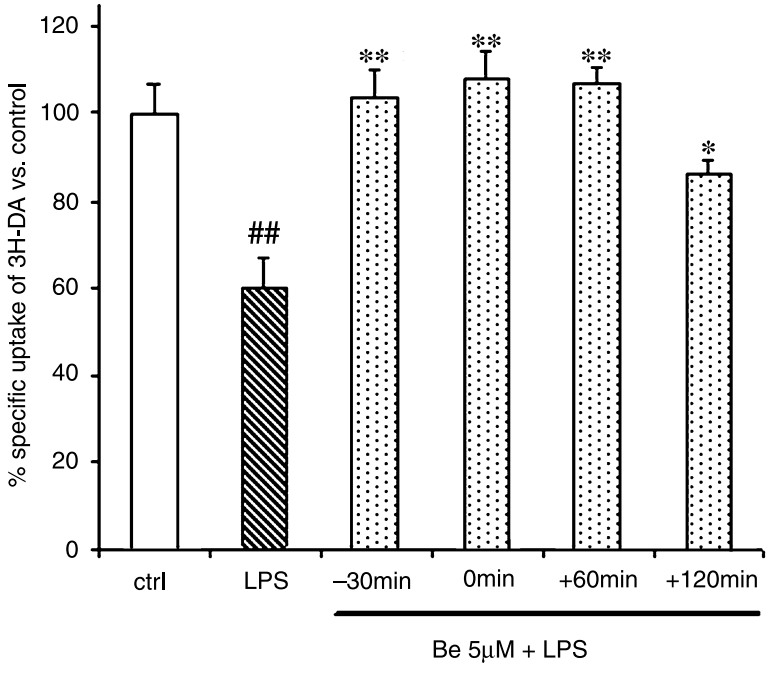

Fig. 3. Effect of post-treatment with baicalein on LPS-induced dopaminergic neurodegeneration. Baicalein (5 μM in final concentration) was added 30 min prior to or 0–120 min after LPS (10 ng/ml) treatment. The experiment of DA uptake was performed seven days later. Data were expressed as the percentage of the control group and presented as mean ± SEM of two individual experiments and each performed in triplicate. $^{\#\#}p < 0.01$ compared with vehicle control and $^{*}p < 0.05$; $^{**}p < 0.01$ compared with the group of LPS-treated alone

The neuroprotective effect of baicalein was also examined at a post-treatment scheme. To this end, neuron-glial cultures were treated with baicalein (5 μM) 30 min prior to, or at the mean time, or 60, 120 min after LPS (10 ng/ml) application. Seven days later, the experiment of DA uptake was performed. As shown in Fig. 3, significant neuroprotection was observed in cultures with baicalein medication, up to 120 min after the administration of LPS. Interestingly, baicalein, with 60 min post-treatment, still completely blocked the toxicity of LPS revealed by being devoid of significant difference compared with the group of 30 min pretreatment.

Effect of baicalein on LPS-induced microglial activation

To elucidate the underlying mechanism of the neuroprotective activity of baicalein, we investigated the effect of baicalein on the LPS-induced microglial activation revealed by OX-42 immunostaining and nitric oxide assay. In normal conditions, microglia keeps in resting state and morphologically presents as non-ramified cells without processes and relatively smaller in size (Fig. 4A).

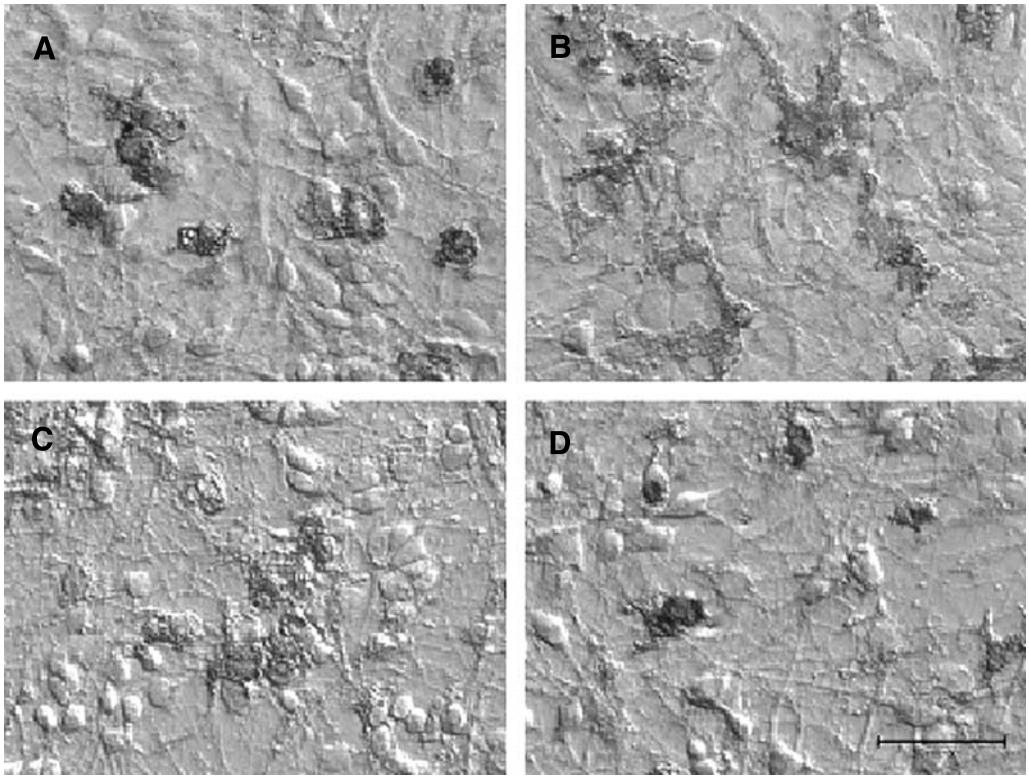

Fig. 4. Effect of baicalein on LPS-induced microglial activation in primary mesencephalic neuron-glial cultures. Baicalein (5 μM) or vehicle was added 30 min prior to LPS (10 ng/ml) treatment. The cultures were fixed with 3.7% formaldehyde 21 h after LPS treatment for further OX-42 immunostaining, which is biomarker of murine microglia. The images were captured under converted phase-contract microscope equipped with SPOT-2 CCD at a magnificent of 200×. The bar showed at the bottom of left panel equals to 200 μm. **A** control group (vehicle alone); **B** LPS-treated alone; **C** baicalein (5 μM)-treated alone; **D** Be (5 μM) with LPS

After treated with LPS (10 ng/ml) for 20 hours, OX-42 immunostained microglia showed a highly differentiated state with enlarged cell body and stout processes (Fig. 4B). Interestingly, treatment with baicalein (5 μM) completely blocked LPS-induced activation of microglia (Fig. 4D). In addition, baicalein alone (without LPS) showed no evidence to activate microglia (Fig. 4C).

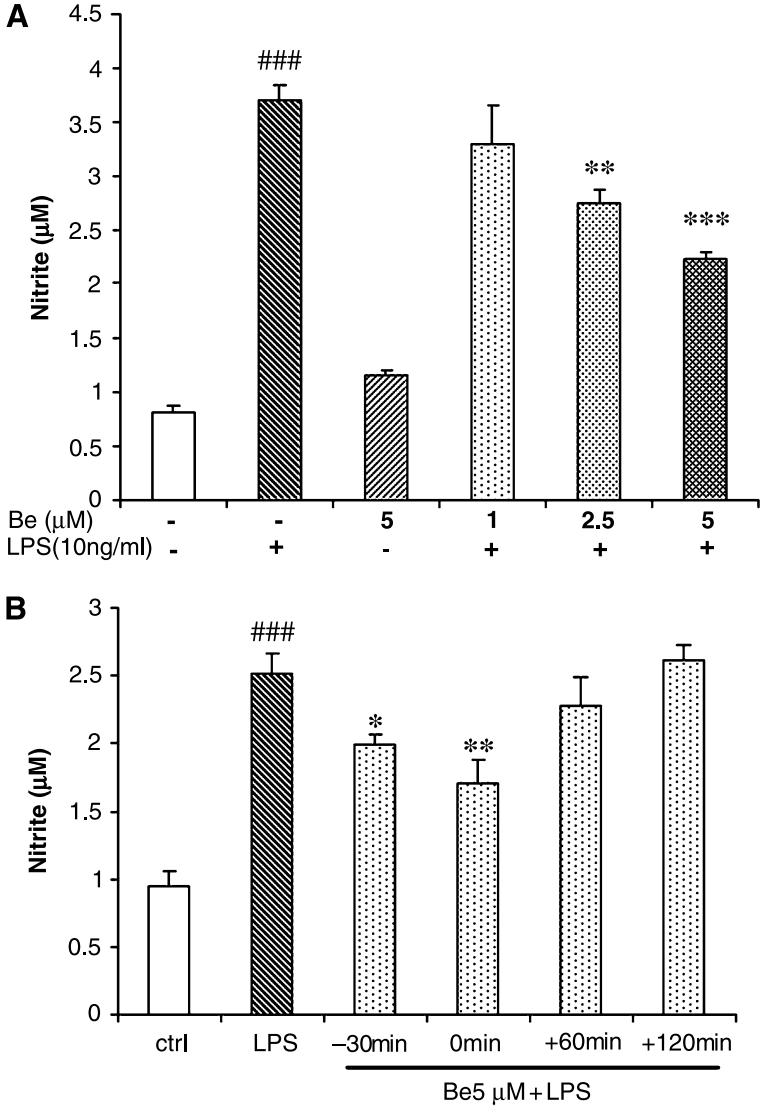

Fig. 5. Effect of baicalein on LPS-induced nitric oxide production in primary mesencephalic cultures. **A** To study the relationship of concentration-effect, indicated concentrations of baicalein were applied 30 min prior to LPS (10 ng/ml) treatment, and **B** to study the effect of post-treatment, baicalein (5 μM) was applied 30 min prior to or 0–120 min after LPS (10 ng/ml) treatment. Fifty micro liters of supernatant per well was collected at 48 h for nitric oxide assay. The production of NO was presented as the accumulated level of nitrite in the supernatant by the method of Griess reaction. The experiments were repeated 3 times and the similar pattern of inhibition was observed. The data were presented as mean ± SEM ($n = 4$). $^{###}p < 0.001$ compared with control group and $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the group of LPS-treated alone

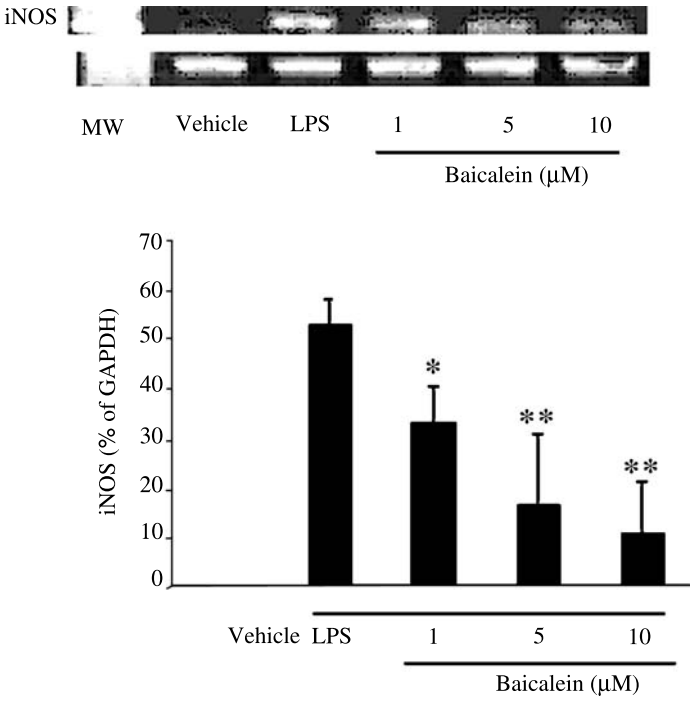

Fig. 6. Effect of baicalein on LPS-induced iNOS mRNA expression. BV-2 cells (5×10^5/well in 1 ml medium) were pretreated with or without baicalein for 30 min prior to treatment with LPS (10 ng/ml) for 3 hr. Then iNOS mRNA expression was detected with RT-PCR. Top: One experiment, representative of three, is shown. Bottom: The intensity of the bands was measured by densitometry and the value for LPS-induced iNOS mRNA taken as 100%. Means ± SEM of the percentages obtained in three independent experiments are shown. $^*p < 0.05$, compared with LPS-treated cultures. Pretreatment with baicalein significantly decreased iNOS mRNA expression

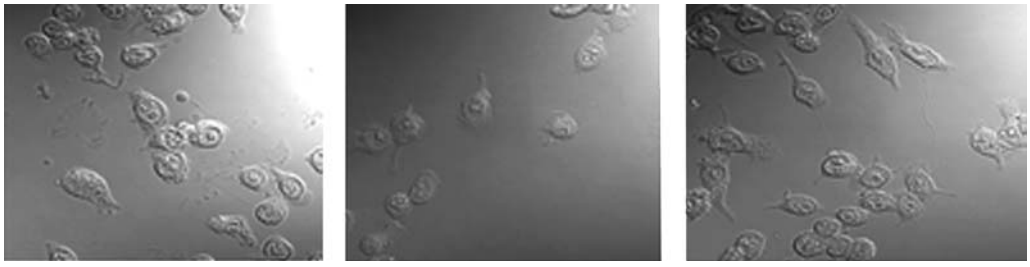

Fig. 7. Effect of baicalein on LPS-induced superoxide production in BV-2 cell cultures. Detection of intracellular superoxide production was done by a dihydroethidium staining method. BV-2 cells (5×10^4) were cultured on coverslips. After overnight incubation, individual cell cultures were treated with various reagents for two and a half hours. For the baicalein-treated group, baicalein was always given 30 min prior to LPS stimulation. Cultures were then stained with dihydroethidium at the concentration of 2 μM for 20 min. Then, the cells were washed with PBS and subsequently fixed with 10% buffered formalin. Production of superoxide was measured as increase of the florescence signals. Images were from one of the three independent experiments with the similar results

Effect of baicalein on LPS-induced NO production and iNOS expression

Accumulation of nitrite, an indicator of LPS-stimulated production of NO, was determined at 48 hr following LPS stimulation. As shown in Fig. 5A, treatment with LPS produced a robust increment of NO level and pretreated with baicalein dose-dependently reduced such an increment by 30–41% (Fig. 5A). In addition, baicalein alone showed no hint to facilitate the production of NO in primary neuron-glial cultures.

In the post-treatment scheme, only 30 min and 0 min pretreatment significantly attenuated nitrite accumulation, while 60 and 120 min post-treatment failed to show obvious effect.

To pursue the underlying mechanism of baicalein's inhibition on NO production, the expression of iNOS mRNA in BV2, a murine microglial cell line, was evaluated by the method of RT-PCR. As shown in Fig. 6, baicalein, significantly inhibited LPS-elicited iNOS expression in a dose-dependent manner.

Effect of baicalein on superoxide production

The effect of baicalein on LPS-stimulated production of superoxide was determined in BV-2 cultures. As shown in Fig. 7, LPS induced significant increase of florescence signals in BV-2 cells compared with control cells. While, baicalein, at the concentration of 5 μM, significantly attenuated LPS-induced increase of florescence signals.

Discussion

In the mesencephalic mixed neuron-glial culture, stimulation of microglial activation with an inflammagen lipopolysaccharide (LPS) induces the production of neurotoxic factors, including tumor necrosis factor-alpha (TNFα), interleukin 1-beta (IL-1β), NO, superoxide, and consequent degeneration of dopaminergic neurons (Liu et al., 2000; Gayle et al., 2002; Gao et al., 2002a, b). Therefore, the *in vitro* model of inflammation-mediated dopaminergic neurodegeneration provides a powerful tool for mechanistic studies and for the identification of potential therapeutic agents (Gao et al., 2003). In this study, we first report that baicalein, a kind of flavonoid extracted from Chinese herbal medicine *Scutellaria baicalensis* Georgi, potently protects dopaminergic neurons against LPS-induced degeneration. Baicalein is more potent, which provides a complete protect against the toxicity of LPS, than other compounds previously tested in our laboratory, such as Gö6976 and naloxone (Jeohn et al., 2002; Liu et al., 2002b). The neuroprotective effect of baicalein may be related to its ability to inhibit the production of neurotoxic factors such as NO, and especially superoxide, by activated microglia.

Scutellaria baicalensis is one of the most widely used herbal medicines against various infectious and inflammatory diseases. The herb has been reported to be antipyretic, antibacterial, and antihypertensive, and has been used to treat stroke (Gong and Sucher, 1999). The root of *S. baicalensis* contains a number of flavone derivatives, such as baicalin, baicalein and wogonin

(Zhang et al., 1998). It was found that flavonoid was a novel class of natural free radical scavenger, besides α-tocopherol, ascorbic acid and β-carotene, and most of the flavonoids were more potent antioxidants than α-tocopherol (Cao et al., 1997). Several lines of evidence indicated that baicalein is the most effective component of *S. baicalensis* in antioxidant and/or free radical scavenging properties (Shieh et al., 2000; Gao et al., 1999). Recently, some work has been done on the protective effect of flavonoids on both cardiomyocytes and neurons (Lebeau et al., 2001; Kim et al., 2001a, b; Shao et al., 2002; Gao et al., 1998, 1999, 2001). These studies showed that the high effectiveness of baicalein on scavenging free radicals could protect neuronal cells from oxidative stress. More recently, Lebeau et al. reported that baicalein protected cortical neurons from β-amyloid (25–35) induced toxicity at low concentrations (0.5–1 μM) (Lebeau et al., 2001). In agreement with the above reports, we first found that baicalein also protected mesencephalic dopaminergic neurons in addition to cortical neurons. Concerning the underlying mechanism, we found that baicalein potently inhibited LPS-induced superoxide production in enriched-microglia cultures and NO production in neuron-glial cultures. These observations further confirmed the contribution of ROS scavenging/antioxidant properties of baicalein to its neuroprotective effect. Our finding also helps to understand the inflammatory mechanism of neurodegenerative diseases.

Recently, converging evidence lends support to the neuroimmune hypothesis of PD's etiology (Liu et al., 2002a; Vila et al., 2001; Kuhn et al., 1997). The core of the hypothesis is that inflammatory response of activated glial cells, especially microglia, may participate in the degeneration of dopaminergic neurons. Several lines of evidence indicated that the dopaminergic system in SNpc and striatum is most susceptible to inflammation-mediated damage (Vila et al., 2001). Interestingly, aside from the dramatic loss of dopaminergic neurons, the SNpc is also the site of a glial reaction in both PD patients and experimental models of PD (Forno et al., 1992; McGeer et al., 1988; Kohutnicka et al., 1998; Sheng et al., 1993). In normal brains, neither resting astrocytes nor microglial cells are evenly distributed (Damier et al., 1993; Lawson et al., 1990). For example, density of microglia in the substantia nigra is remarkably higher compared to other midbrain areas and brain regions such as hippocampus (Kim et al., 2000). Another characteristics of microglia is their activation at a very early stage in response to injury (McGeer and McGeer, 1995; Kreutzberg, 1996; Gehrmann et al., 1995; Giulian, 1999). Microglia activation often precedes reactions of any other cell type in the brain. This is in consistent with our observation that LPS treatment for 20 h induced complete microglial activation in primary neuron-glial cultures. This observation, together with the finding that substantia nigra neurons are much more susceptible to activated microglial-mediated injury, lend support to the idea that gliosis may play an especially meaningful role in Parkinson's disease (Vila et al., 2001). However, it still remains unclear whether gliosis formation is primary event or secondary to the neuronal death. Nevertheless, activated glial cells, especially microglia, can produce a variety of noxious compounds including reactive oxygen species, reactive nitrogen species, pro-inflammatory prostaglandins, and cytokines. Among the array of reactive species, lately, the lion's share of attention has been

given 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 (Liberatore et al., 1999; Torreilles et al., 1999; Vila et al., 2001).

Excessive accumulation of NO has long been known to be toxic to neurons (Bronstein et al., 1995; Dawson et al., 1994; Jeohn et al., 1998; Liu et al., 2002a; Chao et al., 1992). The over-production of free radicals is especially deleterious to neurons (Floyd, 1999; Cadet and Brannock, 1998). However, when NO meets with superoxide, a kind of more deadly production formed, namely, peroxynitrite, which is a potent oxidant and nitrating agent capable of attacking and modifying proteins, lipids and DNA as well as depleting antioxidant defenses (Torreilles et al., 1999; Estevez and Jordan, 2002). In fact, a recent study has identified peroxynitrite as a key mediator of neurotoxicity induced by LPS or β-amyloid peptide (1–42)-activated microglia (Xie et al., 2002), which is consistent with our findings that LPS induced a robust production of both NO and superoxide in microglia cultures. In our present investigation, aside from the inhibition of superoxide, baicalein also potently inhibited NO production by 40% in neuron-glial cultures. Comparatively, it is one of the most potent inhibitors of NO production among tested agents in our laboratory, such as naloxone, Gö6976, dextromethorphan (Jeohn et al., 2002; Liu et al., 2000, 2002b, 2003). Thus, our explanation to the powerful neuroprotective effect of baicalein is derived from its dual functions in inhibiting both superoxide and NO production and consequent formation of lethal peroxynitrite.

In this study, baicalein appears to be significantly more potent in inhibiting LPS-induced superoxide production than the production of NO and TNFα (Figs. 5 and 6). In fact, when mesencephalic neuron-glia cultures stimulated with very low concentrations of LPS (<1 ng/ml), superoxide, other than NO and TNFα, is the only signal detectable and appears to mediate LPS-induced dopaminergic neurotoxicity (Gao et al., 2002a). Furthermore, in our *in vitro* inflammatory model, superoxide production was always observed at very early phase, i.e., 10 to 20 min, while TNFα became detectable 2 h and NO 12 h after LPS treatment. So, we speculate that aside from direct deleterious effect to neuron, superoxide may also play a pivotal role in initiating inflammatory cascade including the production of TNFα 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. We will further translate the in vitro study to animal models to investigate the clinical promise of baicalein.

Using a sensitive fluorescence dihydroethidium, we found LPS evoked robust superoxide signal in murine microglial cell line, BV2 and baicalein treatment obviously depleted superoxide signal. Limited by the methodology of superoxide assay, the present study failed to provide direct answer whether the phenomena arises from scavenging LPS-induced ROS production, or directly preventing ROS production, or both. Nevertheless, several groups had confirmed its ROS scavenging action by electron spin resonance (ESR) spectrometer in the *in vitro* system (Cao et al., 1997; Shieh et al., 2000;

Gao et al., 1999). Moreover, baicalein exerts inhibitory effects on several oxidases, such as xanthine oxidase (Shieh et al., 2000), 12-lipoxygenase (Dailey and Imming, 1999). Therefore, we speculate that both ROS scavenging and antioxidant property play roles in superoxide reduction.

Our results show that another significant characteristic of baicalein is the inhibitory effect on overproduction of NO induced by LPS. It remains unclear to the mechanism of NO inhibition of baicalein. Recently, Chen et al. reported that baicalein inhibited NO production in a concentration-dependent manner in RAW 264.7 macrophages, and the decrease in NO production was in parallel with the inhibition of LPS-induced iNOS gene expression (Chen et al., 2001). In our present study, we provided direct evidence that baicalein also inhibited iNOS expression in microglia. Accumulating evidences indicated that iNOS was the most important contributor to NO production in the brain after inflammatory assault, compared with the other isoforms of NOS, i.e., eNOS and nNOS (Dawson et al., 1994; Liberatore et al., 1999; Iravani et al., 2002; Castano et al., 1998). Therefore, the agents exerting inhibitory effect on iNOS will definitely be beneficial to protect neurons. Our present results also suggested several lines of hints in the respect of iNOS inhibition of baicalein. First, baicalein do inhibited LPS-induced NO over-production in the pretreatment scheme, suggesting its preventive potential; second, baicalein still significantly inhibited NO production given 2 h after LPS treatment, suggesting its therapeutic potential. Previous work in our laboratory suggested that iNOS mRNA became detectable 2 h after LPS challenging and reached the peak at 12 h (Jeohn et al., 2000; Chang et al., 2000).

In summary, our work showed a potent neuroprotective effect of Chinese herbal extract baicalein on dopaminergic neurons in the in vitro chronic inflammatory model. Both ROS inhibitory and anti-inflammatory properties contribute to the neuroprotective effect of baicalein. The further study in the in vivo animal model is highly urgent to confirm its effectiveness. 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, the therapeutic implications of baicalein may thus prove successful in slowing or even halting further degeneration of PD targeting a specific aspect of the glial-related cascade of deleterious events.

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