Catalpol protects dopaminergic neurons from LPS-induced neurotoxicity in mesencephalic neuron-glia cultures

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Abstract

Inflammation plays an important role in the pathogenesis of Parkinson’s disease (PD). Microglia, the resident immune cells in the central nervous system, are pivotal in the inflammatory reaction. Activated microglia can induce expression of inducible nitric-oxide synthase (iNOS) and release significant amounts of nitric oxide (NO) and TNF-α, which can damage the dopaminergic neurons. Catalpol, an iridoid glycoside, contained richly in the roots of Rehmannia glutinosa, was found to be neuroprotective in gerbils subjected to transient global cerebral ischemia. But the effect of catalpol on inflammation-mediated neurodegeneration has not been examined. In this study, microglia in mesencephalic neuron-glia cultures were activated with lipopolysaccharide (LPS) and the aim of the study was to examine whether catalpol could protect dopaminergic neurons from LPS-induced neurotoxicity. The results showed that catalpol significantly reduced the release of reactive oxygen species (ROS), TNF-α and NO after LPS-induced microglial activation. Further, catalpol attenuated LPS-induced the expression of iNOS. As determined by immunocytochemical analysis, pretreatment by catalpol dose-dependently protected dopaminergic neurons against LPS-induced neurotoxicity. These results suggest that catalpol exerts its protective effect on dopaminergic neurons by inhibiting microglial activation and reducing the production of proinflammatory factors. Thus, catalpol may possess therapeutic potential against inflammation-related neurodegenerative diseases.

Keywords: Catalpol; Inflammation; Microglia; Neurons; Lipopolysaccharide; Neurodegeneration

Introduction

Parkinson’s disease (PD) is a common neurodegenerative disorder characterized by cardinal features, including resting tremor, slowing of movement, rigidity and postural instability. The disease is characterized by a progressive and selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Olanow and Tawwon, 1999). Although the etiology of the disease remains elusive, increasing evidence from clinical and experimental animals has suggested that glia-mediated inflammation plays a pivotal role in the neuronal loss in PD (Hirsch et al., 1998; Gao et al., 2002). Postmortem studies have shown that there are a large number of activated microglia in the SN of PD patients. A robust activation of microglia was also found in both 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- and rotenone-induced Parkinsonism animal models (Kurkowska-Jastrzebska et al., 1999; Orr et al., 2002). Injection of bacterial lipopolysaccharide (LPS) into rat SN and the ensuing microglial activation has been shown to induce dopaminergic neurodegeneration (Liu et al., 2000b; Iravani et al., 2002). Microglia, the resident immune cells of the brain, are sensitive to infection or changes in their microenvironments and readily become activated in response to infection or injury. Upon CNS injury, activated microglia release a variety of factors, including cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), free radicals, nitric oxide (NO) and superoxide (Liu et al., 2002; Wu et al., 2002; Kremlev et al., 2004), which have been thought to increase neurotoxicity to dopaminergic neurons.

The free radical NO produced by activated microglia has many physiological functions. In the brain it serves as a neurotransmitter and a second messenger molecule mediating a
variety of neuronal functions. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). There are three major isoforms of this enzyme in the central nervous system (CNS). eNOS and nNOS are isoforms of constitutive NOS, which are regulated by calcium and are responsible for intermittent production of small amounts of NO. Inducible NO synthase (iNOS) is calcium-independent. It is induced by a variety of inflammatory stimuli such as cytokines. It has been reported that expression of iNOS during inflammation in the CNS plays a role in the neurodegeneration in PD (Dawson et al., 1994; Dawson and Dawson, 1996). Up to now, a great number of compounds targeting these perilous factors have been proven to be effective in the animal models. However, due to unbearable side effects at higher doses, there is no agent which has been established for use in humans. There is now an increasing interest in focusing on natural products. Some natural products separated from traditional Chinese herbal medicines have been shown to be neuroprotective in vitro and in vivo in our laboratory (Jiang et al., 2004; Li et al., 2004a, 2005).

Iridoid glycoside is an iridoid and has a range of biological abilities including purgative, anti-tumor and sedative activities. Catalpol, an iridoid glucoside separated from the roots of Rehmannia glutinosa, has been reported to attenuate apoptosis induced by H$_2$O$_2$ in PC12 cells in vitro (Jiang et al., 2004), protect hippocampal CA1 region neurons from death through anti-oxidative ability and reduce cognitive impairment significantly (Li et al., 2004a, 2005). Because inflammation and oxidative stress are important players in the pathogenesis of PD, the neuroprotective action of catalpol has been measured in primary mesencephalic neuron-glia cultures. The aim of the present study was to explore whether catalpol could protect the dopaminergic neurons from toxicity induced by LPS and find the possible mechanisms of the neuroprotection.

**Materials and methods**

**Reagents**

Catalpol was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), then was diluted in physiological saline for treatment. The monoclonal anti-TH antibody and anti-CD11b antibody were purchased from Chemicon. SABC compound kits were purchased from Sino-American Biotechnology Company. 2′,7′-Dichlorofluorescein diacetate was obtained from Beyotime. Lipopolysaccharide (LPS) (E. coli, 055:B5) was purchased from Sigma. Tissue culture media and fetal bovine serum were obtained from Gibco.

**Primary mesencephalic mixed neuron-glia cultures**

Neuron-glia cultures were prepared from the ventral mesencephalic tissues of embryonic day 14/15 mice, as described previously (Liu et al., 2000a). Briefly, dissociated cells were seeded at $1 \times 10^5$/well to poly-$\alpha$-lysine coated 24-well plates. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO$_2$ and 95% air in Dulbecco’s modified Eagle’s medium/ nutrient F12 (DMEM/F12) containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, 1.2 g/l sodium bicarbonate and 2 mM L-glutamine. Seven-day-old cultures were used for treatment, and were made up of 12% microglia, 48% astrocytes, and 40% neurons, of which 2 to 3% were tyrosine hydroxylase-immunoreactive (TH-IR) neurons.

**Primary microglia culture**

Microglia-enriched cultures were prepared from the whole brains of 1-day-old mice as previously described (Li et al., 2004b). Briefly, the brains were dissociated by a mild mechanical trituration in DMEM/F12 with 10% FBS. The isolated cells were seeded at $1 \times 10^6$ to poly-$\alpha$-lysine coated 35-cm$^2$ T-flasks. Upon reaching confluence (12–14 days), microglia were detached from the flasks by mild shaking for about 6 h at 190 rpm. Cells were seeded at $1 \times 10^5$/well to 96-well plates. After 24 h, the cultures were used for experiments. Purity of the enriched microglia was >95%, as determined by complement receptor 3 (CD11b) immunocytochemistry staining.

**Immunocytochemistry**

Immunostaining was performed as previously described (Zhou et al., 2005). Dopaminergic (DA) neurons were recognized with a rat monoclonal anti-TH antibody (1:300, Chemicon). Briefly, formaldehyde-fixed cultures were treated with 1% hydrogen peroxide followed by sequential incubation with blocking solution for 30 min. Cells were incubated overnight at 4 °C or 37 °C for 2 h with primary anti-TH diluted in antibody diluent. The bound primary anti-TH antibody was visualized by incubation with addition of biotinylated secondary antibody, followed by the ABC reagents and color was developed with 3, 3′-diaminobenzidine. For morphological analysis, the images were recorded with an inverted microscope (OLYMPUS CK40) connected to a camera. For each condition, quantification of the nigral TH-positive neurons was performed by visually counting the number of mesencephalic TH-positive neuronal cells with clearly visualized cell body outlines under a microscope in three to four wells in a blind fashion. Identification of microglia was performed using rat monoclonal anti-CD11b and a goat anti-rat IgG (1:200). Cells were visualized using an inverted microscope.

**Nitrite, TNF-$\alpha$ and inducible nitric oxide synthase (iNOS) assays**

Mensencephalic neuron-glia cultures were pretreated with catalpol (0.05–0.5 mM) for 30 min, then treated with LPS for additional 24 h. The production of nitric oxide (NO) was determined by measuring the accumulation of nitrite in the medium reaction with the Griess reagent as described previously (Green et al., 1982). Briefly, after 24 h of treatment with LPS (10 μg/ml) and the various concentrations of catalpol, the culture supernatants were collected and mixed with an equal volume of Griess reagent (0.1% N-(1-naphthil) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H$_3$PO$_4$). The absorbance was measured at 545 nm. A standard curve using...
NaNO₂ was generated in each experiment for quantification. The amount of tumor necrosis factor-α (TNF-α) released into the medium was measured with a mouse TNF-α enzyme-linked immunosorbent assay kit from R and D Systems according to the manufacturer’s instructions. The activities of iNOS were determined by using commercially available kits (Nanjing, Jiancheng, Bioengineering). All procedures completely complied with the manufacturer’s instructions. The assay for iNOS activity depends on its ability to catalyze Arginine (Arg) to form NO, which can further react with nucleophilic substance to produce chromophoric compound that has the largest absorbance at 530 nm.

Assay of intracellular reactive oxygen species (ROS)

ROS were measured with the non-fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) as previously described (Liu et al., 2001). DCFH-DA passively diffuses into cells and is deacetylated by esterases to form nonfluorescent 2',7'-dichlorofluorescein (DCF). In the presence of ROS, DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cells. Microglia collected from culture flasks were seeded at a density of 1×10⁵/well in 96-plate. One day after seeding, the culture wells were pretreated with catalpol (0.05–0.5 mM) 30 min, then treated with LPS (10 μg/ml) for an additional 24 h. To obtain dissociated microglia for the ROS assay, culture medium was first removed and the cells were washed three times with PBS. DCFH-DA, diluted to a final concentration of 10 μM with DMEM/F12, was added to cultures and incubated for 20 min at 37 °C. The fluorescence was read at 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (Genios, TECAN). The increase in fluorescence compared to control was viewed as the increase of intracellular ROS.

Cell viability assay

The effect of catalpol on the viability of LPS-induced microglia was analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983; Zhou et al., 2005). After treatment with LPS and corresponding concentrations of catalpol for 24 h, MTT (5 mg/ml) was added to the microglia cultures. Cells were then incubated at 37 °C for an additional 3 h. At the end of the incubation period, the supernatants were removed, and 100 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formed blue formazan. Absorbance was read at 570 nm on a microplate reader. Cell viability was expressed as a percentage of the LPS-treated culture.

Statistical analysis

All data were expressed as mean±SEM and evaluated by t test. A value of p<0.05 was considered statistically significant.

Results

Catalpol dose-dependently protects dopaminergic neurons against LPS-induced neurotoxicity

Mesencephalic neuron-glia cultures were used to evaluate the effect of different doses of catalpol on LPS-induced dopaminergic neurodegeneration. Mesencephalic neuron-glia cultures were pretreated with desired concentrations of catalpol for 30 min, then stimulated with LPS (10 μg/ml) for additional 24 h. The degeneration of DA neurons was determined by counting the number of TH-positive neurons and morphological inspection. Immunocytochemical analysis for TH-positive neurons demonstrated that application of LPS induced a significant decrease in the number of dopaminergic neurons. Treatment with catalpol (0.05–0.5 mM) could effectively reduce the degeneration of TH-positive cell bodies in midbrain (Fig. 1). Meanwhile, morphological inspection revealed that LPS treatment not only decreased the number of TH-positive neurons, but there was an apparent inhibitory effect on the outgrowth of neurites. These characteristics were reversed by catalpol in a dose-dependent manner. Treatment with catalpol (0.5 mM) alone had no effect on the morphology or numbers of TH-positive neurons in the midbrain (Fig. 2).

Effect of catalpol on microglial activation and the production of proinflammatory and neurotoxic factors

Since activated microglia were observed during the pathogenesis of PD and had been thought to play an important role in the progression of neurodegeneration, the effects of catalpol on LPS-induced microglial activation were examined in the brain. LPS induced activated microglial release of more neurotoxic factors and destroyed the dopaminergic neurons (Orr et al., 2002). To investigate whether catalpol protected DA neurons by inhibiting microglial activation, the effects of catalpol on LPS-induced microglia were analyzed for...
immunostaining by CD11b and the release of ROS and nitric oxide was measured.

Microglial activation was determined by immunostaining with complement receptor 3 (CD11b) antibody. In control culture, most microglia had a light brown stain, whereas LPS-treated neuron-glia cultures showed a significant increase in CD11b-immunoreactivity. In addition, the microglial activation was characterized by a significant change in the morphology. The CD11b-positive microglial cell bodies became larger and irregular. However, pretreatment with catalpol significantly attenuated the LPS-induced microglial activation. Catalpol alone did not elicit any significant changes in the microglia (Fig. 3). Moreover, pretreatment with catalpol decreased the number of microglia in a dose-dependent manner (Fig. 4). The levels of nitrite, an indicator of NO production, were measured in neuron-glia cultures. As shown in Fig. 5A, pretreatment with catalpol (0.05–0.5 mM) for 30 min had dose-dependent inhibitory effects on LPS-induced production of NO in neuron-glia cultures. A moderate reduction in the release of TNF-α by LPS-treated neuron-glia cultures was observed for pretreatment with catalpol (Fig. 5B). Intracellular ROS may act as second messengers in regulating LPS-stimulated production of neurotoxic factors. To determine if the neuroprotective effect of catalpol is due to a reduction in ROS production, the levels of nitrite, an indicator of NO production, were measured in neuron-glia cultures. The number of surviving microglia was quantified. Data are the mean ± SEM of three independent experiments. *p<0.01, **p<0.001 compared with LPS-treated cultures.
intracellular ROS production was measured in microglia-enriched cultures by using DCFH-DA. The ROS level was significantly increased by LPS treatment. The results showed that pretreatment with catalpol (0.05–0.5 mM) dose-dependently attenuated LPS-induced ROS production in microglia-enriched cultures (Fig. 5C).

Pretreatment with catalpol inhibits LPS-induced iNOS expression in mesencephalic neuron-glia cultures

NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). The inducible NOS (iNOS) can release high levels of NO for extended periods of time. It has been reported that expression of iNOS during inflammation in CNS plays a role in the neurodegeneration in PD (Hunot et al., 1996; Arimoto and Bing, 2003). The assay for iNOS activity depends on its ability to catalyze Arginine to form NO, which can further act with nucleophilic substance to produce a chromophoric compound that has the largest absorbance at 545 nm. Measurement of iNOS expression demonstrated that pretreatment with catalpol dose-dependently attenuated LPS-induced iNOS expression in neuron-glia cultures (Fig. 6).

Discussion

It has been increasingly evident that inflammation plays an important role in a variety of neurodegenerative disorders. LPS treatment has been used extensively in inflammation studies (Wang et al., 2005; Zhou et al., 2005). It has been shown that LPS activates microglia and exerts neurocytotoxic effects on dopaminergic neurons in the nigrostriatal system both in vitro and in vivo (Becher et al., 1996; Iravani et al., 2002; Peng et al., 2005). Therefore, LPS-treated mesencephalic neuron-glia cultures provide a suitable model for studying the role of inflammation in Parkinson’s disease. Bacterial LPS activates microglial cells because the cells express the LPS-binding receptor CD14 (Becher et al., 1996), enabling the cellular response. PD is a neurodegenerative disease characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Therefore, the neuroprotective effects provided by some agents have been evaluated by counting the dopaminergic neurons in the substantia nigra subfield in many studies. In this study, as determined by counting the TH-positive neurons and morphological inspection, the results showed that, on the one hand, LPS induced a decrease in the number of TH-positive cells in mesencephalic neuron-glia cultures. On the other hand, the results demonstrated that...
pretreatment with catalpol could protect dopaminergic neurons from LPS-induced neurotoxicity in the midbrain, and the neuroprotective efficacy of catalpol was dose-dependent.

The hallmark of neuroinflammation is the activation of microglia, the resident brain immune cells that release cytokotic factors, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), nitric oxide (NO) and reactive oxygen species (ROS) (Liu et al., 2002; Wu et al., 2002), which can cause neurodegeneration (Hirsch et al., 1998; Iravani et al., 2002; Hunot et al., 1996). Brain microglia can induce expression of iNOS and release significant amounts of NO within hours of LPS induction. Generation of NO in response to various stimuli has been studied intensively and is implicated in a variety of neuroinflammatory and neurodegenerative disorders. It has also been shown that microglia are a major source of iNOS in the SN after LPS exposure and the activation of microglia by LPS causes neuronal death in mixed neuron-glia cocultures with mechanisms involving glial iNOS expression and release of NO (Le et al., 2001). This study demonstrated that the neuroprotective effects of catalpol were closely correlated with suppression of microglial activation. Pretreatment with catalpol showed a moderate inhibitory effect on LPS-induced production of NO and attenuated the expression of iNOS in a dose-dependent manner.

ROS and NO produced by activated microglia are two factors that have been implicated in the mediation of inflammation-induced neurotoxicity (Farber, 1994; Arimoto and Bing, 2003). ROS, including superoxide anion, hydroxyl radical, lipid hydroperoxides and their byproducts (e.g., hydrogen peroxide) may play a dual role in neurodegenerative diseases. While intended to kill invading pathogens, ROS generated by activated microglia are also toxic to neurons by inducing lipid peroxidation, DNA fragmentation and protein oxidation (Farber, 1994). Furthermore, ROS can activate diverse downstream signaling molecules (Rosenberger et al., 2001). In this study, the results demonstrated that LPS increased intracellular ROS production in microglia-enriched cultures. This observation was consistent with the report by Gao et al. (2003) which indicated that LPS increased the production of intracellular ROS. More importantly, pretreatment with catalpol dose-dependently attenuated LPS-induced intracellular ROS.

In conclusion, the present study showed that catalpol, an iridoid glucoside, could effectively attenuate LPS-induced microglial activation and subsequent dopaminergic neurotoxicity. The inhibitory effects of catalpol on LPS-induced microglial activation depended on two mechanisms. Firstly, catalpol attenuated the expression of iNOS. Secondly, pretreatment with catalpol could inhibit the production of TNF-α, NO and the intracellular ROS. These observations suggested that catalpol might be a potential therapeutic agent for the treatment of inflammatory-related neurodegenerative disorders.

References


