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Effects of catalpol on mitochondrial function and working memory in mice after lipopolysaccharide-induced acute systemic inflammation

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Abstract

The aim of this study was to investigate whether catalpol could facilitate recovery from lipopolysaccharide (LPS)-induced cognitive deficits and protect brain mitochondrial function from LPS-induced acute systemic inflammation. In the study, except control group, mice were challenged with a single dose of LPS (100 μg/mouse, i.p.) to mimic an acute peripheral infection. The results showed that LPS enhanced nuclear factor kappa B (NF-κB) activation and induced a loss in mitochondrial integrity as shown by a significant decrease in membrane potential and increase in mitochondrial permeability transition pore opening. Pretreatment with catalpol (10 mg/kg d, i.p.) for 10 d before injection of LPS reversed the memory deficits induced by LPS, protected brain mitochondrial function, and attenuated LPS-induced NF-κB activation. Taken together, these data indicate that catalpol may possess therapeutic potential against LPS-induced acute systemic inflammation by attenuating NF-κB activation and protecting mitochondrial function in cerebral cortex and hippocampus.

Keywords: Catalpol; Inflammation; Mitochondria; NF-κB; Lipopolysaccharide

Introduction

Increase in life expectancy has resulted in an increase in the prevalence of age-dependent diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). The symptoms of these diseases include progressive memory loss and impairment in spatial and perceptual recognition, as well as in daily living. Brain inflammation probably plays an important role in the pathogenesis of chronic neurodegenerative disorders like AD and PD (Nelson et al., 2002; Liu and Hong, 2003). Neurodegeneration caused by inflammation involves activation of the brain's resident immune cells and the microglia, which produce a large number of pro-inflammatory factors (Pocock and Liddle, 2001; Hanisch, 2002). Lipopolysaccharide (LPS) is a highly conserved cell wall component of gram-negative bacteria that is recognized by the immune system of higher vertebrates as a pathogen-associated molecular pattern (PAMP). LPS binds to the Toll-like receptor 4 (TLR4)/CD14 complex on the surface of mononuclear myeloid cells (Laflamme and Rivest, 2001) and activates the

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transcription factor NF-κB to up-regulate expression of, among other genes, the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. Recently, peripheral immune activation with LPS has been reported to exacerbate neuroinflammation and prolong sickness behavior in aged mice (Godbout et al., 2005a). Florence et al. (2007) have recently shown that acute systemic inflammation caused by LPS induces central mitochondrial damage and mnesic deficit in adult Swiss mice. Intracellular reactive oxygen species (ROS), generated by highly respiring mitochondria, and peroxides are also well documented to serve as intracellular second messengers to induce signal transduction and activate transcription factors such as NF-κB (Haddad et al., 2002; Zhang et al., 2001).

Catalpol, an iridoid glucoside separated from the roots of *Rehmannia glutinosa*, possessing a wide range of biological and pharmacological activity, including anti-tumor, anti-inflammation, and anti-apoptosis properties, has been reported to significantly improve the cognitive impairment in many animal models of neurodegenerative disease (Li et al., 2004, 2005; Zhang et al., 2007) and protect mice brain from oxidative damage and mitochondrial dysfunction induced by rotenone (Mao et al., 2007). Furthermore, a recent study suggests that catalpol is neuroprotective in vitro since it significantly reduced the release of ROS, TNF-α, NO, and iNOS expression after LPS-induced microglial activation (Tian et al., 2006). Because inflammation and mitochondrial dysfunction are important players in the pathogenesis of neurodegenerative diseases, the neuroprotective action of catalpol has been measured in LPS-induced acute systemic inflammation. The aim of the present study was to investigate whether catalpol could facilitate recovery from LPS-induced cognitive deficits and protect brain mitochondrial function.

Materials and methods

Reagents and drugs

Catalpol, separated and purified according to our previous report (Zhang et al., 2007, 2008), was diluted in physiological saline for treatment. LPS (*Escherichia coli*, 055:B5), obtained from Sigma, was dissolved in phosphate-buffered saline (PBS). The commercial kit used for determination of mitochondrial permeability transition pore (MPTP) opening was purchased from GENMED Company (Shanghai, China). NF-κB p65 antibody and 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Beyotime.

Animals and drug treatment

The Kunming mice (obtained from the Experimental Animal Center, Dalian Medical University, China), weighing 35–37 g, were housed in cages in an air-conditioned room with controlled temperature (24 ± 1 °C) for 5 d before the experiment and were maintained on a 12:12 h light cycle (07:00 h on, –19:00 h off). They were allowed free access to food and water. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Dalian Medical University, Dalian, China.

The mice were randomly divided into three groups: control group ($n = 10$), model group ($n = 10$), and therapy group ($n = 10$). Control group mice were injected with sterile physiological saline (i.p.). Model group mice were treated with sterile physiological saline for 10 d, and then they were treated with LPS (100 μg/mouse, i.p.) for the last day. Therapy group mice were treated with catalpol (10 mg/kg body weight, i.p.) for 10 d, and then they were treated with LPS (100 μg/mouse, i.p.) for the last day. Mice were sacrificed after 4 h from the last drug treatment, and their brains were quickly removed. The cerebral cortex and hippocampus were isolated, weighed, frozen on ice, and stored at −80 °C until assay.

Behavioral testing

A water maze (Morris) was constructed to evaluate mice working memory, which consisted of a black circular tank, 100 cm in diameter and 50 cm in depth. The tank was divided virtually into four equal quadrants and an escape platform was hidden 1.5 cm below the surface of the water in a fixed location in the third quadrant of the pool. After 1-d training, a trial was started by placing the mice into the pool close to the rim, facing the wall of the tank in one of the four quadrants. For the experiment, training took place during a 10-d acquisition phase with two sessions of three massed trials administered each day. A single injection of saline or LPS was administered on the test day (d 11) to determine treatment effects on an animal's ability to integrate new information with existing memories to complete a task. To begin each trial, a mouse was pseudorandomly placed in an arm not occupied by the platform facing the wall. Mice were allowed to swim freely for a maximum of 60 s or until the platform was located. After the mouse reached the platform it was required to remain there for 30 s. If the platform was not located during the 60 s, mice were guided to the platform and allowed to remain for 30 s. After completion of three consecutive trials, mice were placed in their home cage under a heat lamp for approximately 10 min. Performance parameters that were determined included swim speed, latency to the platform, and distance swam.

Isolation of brain mitochondria

The cerebral cortex and hippocampus mitochondria were prepared according to the method of Veitch et al. (1992) with modifications. The cerebral cortex and hippocampus were washed two or three times with fresh MSME medium (mannitol, 220 mM; sucrose, 70 mM; Mops, 5 mM; EGTA, 1 mM, pH 7.4). Two milliliters of MSME containing 3 mg of defatted bovine serum albumin (BSA) was added and the mixture was homogenized in a glass/Teflon homogenizer, and then centrifuged at 600*g* for 5 min. The supernatant was filtered through four layers of gauze and spun at 12 000*g* for 5 min to obtain a first mitochondrial pellet, which was washed in 2 ml of MSME and centrifuged at 8000*g* for 5 min. The homogenate pellet was resuspended in 3 ml of MSME (+1 mg of BSA/ml), rehomogenized, and spun again at 600*g* for 5 min. This second supernatant was filtered through gauze and spun at 8000*g* for 5 min to give a second mitochondrial pellet, which was combined with the first for a final wash and centrifugation at 6000*g* for 5 min. The final pellet resuspended in MSME was used for assay.

Protein assay

Protein concentration was measured according to the method of Bradford (1976). BSA was used as standard.

Measurement of ROS formation

ROS were assayed using DCFH-DA as previously described (LeBel et al., 1992). ROS in the cerebral cortex and hippocampus mitochondria were analyzed according to Hamai et al. (2001) with modifications. Briefly, 10 μl of mitochondria (5–10 μg) was suspended in 170 μl HEPES buffer (the composition of the HEPES buffer was: NaCl, 120 mM; KCl, 2.5 mM; NaH_2PO_4, 1.2 mM; $MgCl_2$, 0.1 mM; $NaHCO_3$, 5.0 mM; glucose, 6.0 mM; $CaCl_2$, 1.0 mM; HEPES, 10 mM, pH 7.4) in 96-well plates; 20 μl of 100 μM DCFH-DA was added to each well for a final volume of 200 μl. The fluorescence was read at 485 nm for excitation and 530 nm for emission with a fluorescent plate reader (Genios, TECAN). The increased fluorescence intensity was viewed as the increase of intracellular ROS.

Measurement of MPTP opening with calcein AM

The opening of MPTP was determined by using commercially available kit (GENMED). All procedures completely complied with the manufacture's instructions. MPT pore opening was measured directly using a combination of calcein AM and $CoCl_2$. Calcein AM, which fluoresces on binding with Ca^{2+}, was used to detect transient MPT pore opening in the high-conductance mode of intact cells (Sharov et al., 2007). This was achieved by monitoring changes in mitochondrial Ca^{2+} levels in the presence of $CoCl_2$, which quenched the cytosolic Ca^{2+} signal produced by calcein. The fluorescence of calcein AM was monitored at the emission wavelength of 530 nm, with the excitation wavelength being 488 nm. The extent of Ca^{2+}-induced MPT pore opening was estimated by noting the difference in fluorescence intensity.

Measurement of mitochondrial membrane potential

Changes in mitochondrial membrane potential were monitored in the presence of the fluorescent dye Rhodamine 123 (Rh123) according to Emanus et al. (1986) with modifications. Briefly, 10 μl of mitochondria (5–10 μg) was suspended in 90 μl assay buffer (the composition of the buffer was sucrose, 15 mM; $MgCl_2$, 5 mM; sodium succinate, 5 mM; K_2HPO_4, 5 mM; HEPES, 20 mM, pH 7.4) in 96-well plate and incubated for 30 min in the presence of 20 μl of Rh123 (100 μg/ml). The change of mitochondrial membrane potential was assayed by measuring the fluorescence change in the reaction mixture at an excitation wavelength of 485 nm and an emission wavelength of 538 nm with a fluorescence plate reader (Genios, TECAN). The results were expressed by fluorescence intensity of Rh123.

Measurement of NF-κB activity in mice cerebral cortex and hippocampus

Subcellular location of NF-κB p65 protein was detected using flow cytometric analysis. Briefly, cells were harvested and washed twice with PBS, and fixed in 4% paraformaldehyde for 20 min. Cells were washed for 5 min with PBS containing 0.1% Triton X-100 and incubated with mouse polyclonal antibody against p65 NF-κB for 1 h at 37 °C in the dark. The mouse anti-NF-κB (nuclear-localized signal) antibody recognizes an epitope overlapping the nuclear location signal of NF-κB p65 and therefore selectively recognizes the activated form of NF-κB. The cells were then labeled with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG monoclonal antibody for 30 min in the dark before the flow cytometric analysis. We analyzed 10 000 cells for each subject in the flow cytometric studies. All antibodies were used at the concentrations recommended by the manufacturer.

Measurement of the spleen indices

Mice were sacrificed after 4 h from the last drug treatment, then spleen were quickly removed and weighed. The spleen indices were calculated by the

following formula: spleen indices (g/kg) = spleen weight/body weight.

Statistical analysis

Data were expressed as mean ± S.E.M. and evaluated using one-way ANOVA followed by Student's *t*-test. The criterion of $P<0.05$ was accepted as statistically significant.

Results

Body weights and spleen indices

Body weights were determined 4 h after each injection on each test day. The spleen of each animal was removed and immediately weighed after the last drug injection. The spleen is a critical immune organ, which has been shown to increase in size after immune challenge due to B and/or T cell differentiation and proliferation. Thus, postmortem spleen weights provide a rough estimate of the degree to which LPS, a B cell mitogen, has influenced the immune system. Fig. 1 shows that the body weights of mice treated with LPS were significantly decreased from 40.67 ± 0.81 to 39.38 ± 1.15 (g, $n = 10$) in the model group, but there was no significant change in the control and therapy groups. The changes in the spleen indices of mice are depicted in Table 1. The model group mice had a significantly higher spleen indices than those of the control group ($P<0.05$). Catalpol therapy group mice showed significant decrease in spleen indices ($P<0.01$ vs. model group).

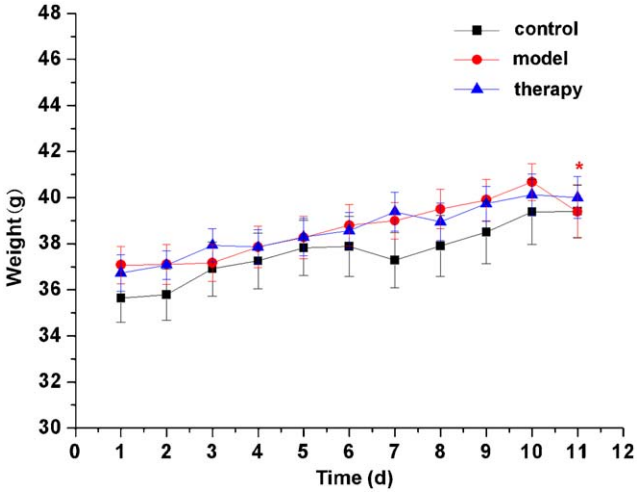

Fig. 1. Group mean changes in body weight 4 h after injection of catalpol (10 mg/kg d) or LPS (100 μg/mouse) or saline vehicle on each test day. *$P<0.05$ vs. day 10. Error bars represent the standard error of the mean (S.E.M.).

Catalpol improved recovery from LPS-induced cognitive deficits

To determine if catalpol could improve recovery from LPS-induced cognitive deficits, mice were subjected to a 10-d catalpol injection regimen. Figs. 2a–c show that the mice were subjected to the 10-d acquisition period and Figs. 2d–f show the effects of catalpol on cognitive deficits in Morris water 4 h after LPS treatment on day 11. The data indicated that the mean distance and latency to find the platform declined progressively during the acquisition phase in all animals, yet there was no significant change of swim speed, indicating that catalpol was not deleterious to mice. Injection of LPS induced cognitive deficits, such as increasing the mean distance and latency ($P<0.05$ vs. control group), but mice pretreated with catalpol recovered from cognitive deficits induced by LPS, decreasing the mean distance and latency ($P<0.05$ vs. model group), yet swim speed was not affected (Fig. 2f). These findings showed that peripheral administration of catalpol, at least to some degree, reversed the memory deficits induced by LPS.

ROS formation

Free radicals, typically generated from mitochondrial respiration, cause oxidative damage of nucleic acids, lipids, carbohydrates, and proteins. To determine if the protective effect of catalpol is due to a reduction in ROS production, the intracellular ROS production was measured by using DCFH-DA. As shown in Fig. 3, LPS exposure significantly increased ROS production in cerebral cortex ($P<0.01$ vs. control group) and hippocampus mitochondria ($P<0.01$ vs. control group). Pretreated with catalpol (10 mg/kg d), DCF production level was restored to the basal level as compared to the model group ($P<0.05$) both in cerebral cortex and hippocampus mitochondria. These data are consistent with the formation of ROS, which may occur in cells exposed to oxidative stress, leading to cellular damage,

Table 1. Effects of LPS and catalpol on spleen indices of mice.

Group	Body weight (g)	Spleen weight (g)	Spleen indices (‰)
Control	39.41 ± 1.15	0.17 ± 0.02	3.83 ± 0.33
Model	39.39 ± 1.15	0.23 ± 0.01	4.99 ± 0.25$^{\#}$
Therapy	40.01 ± 0.91	0.14 ± 0.01	3.25 ± 0.14**

Values are expressed as the mean of ten animals ± S.E.M.
$^{\#}P<0.05$ vs. control group.
**P < 0.01 vs. model group, $n = 10$.

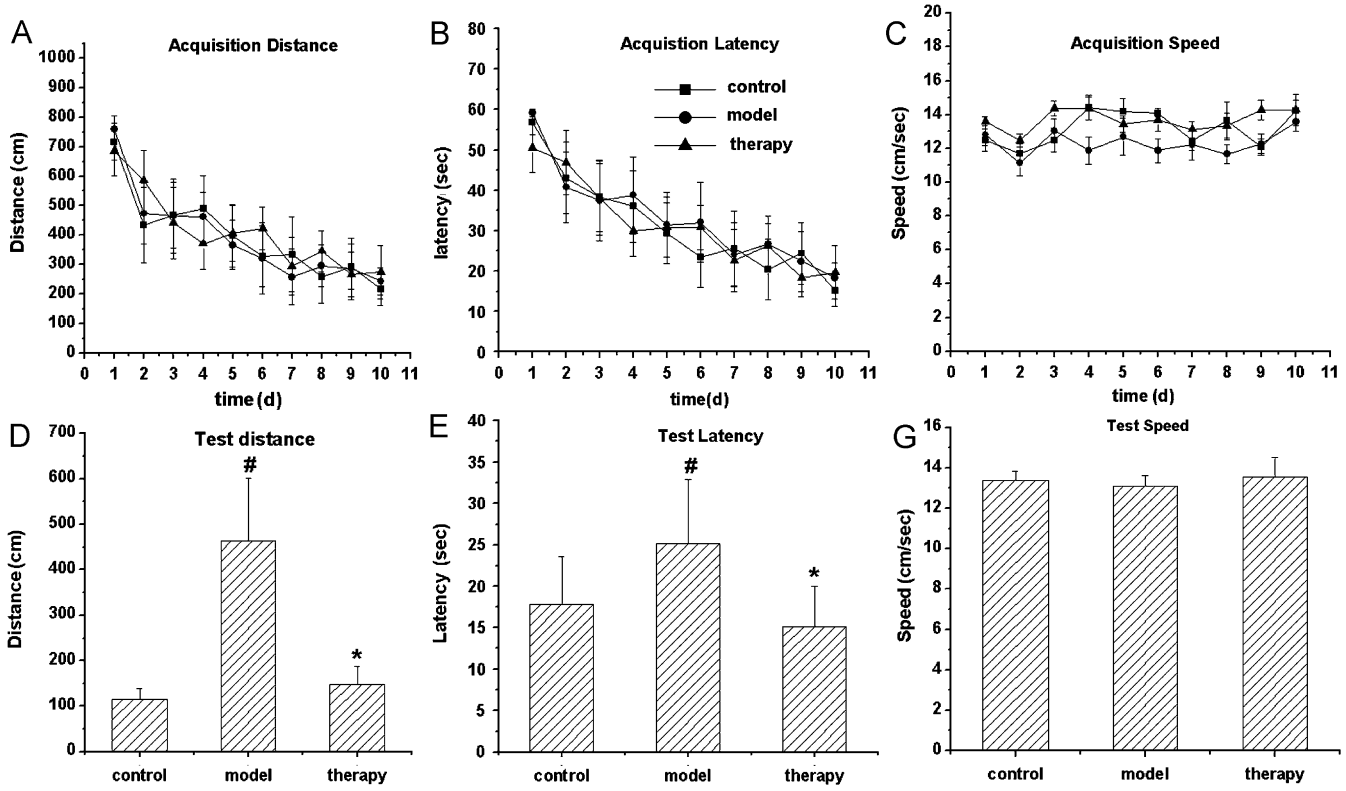

Fig. 2. Effect of catalpol on performance of mice in Morris water. All animals were trained for 10 d. After the 10-d acquisition training, mice were injected with saline or LPS and performance in Morris water was evaluated 4 h later. Performance of acquisition and test phase was shown in mean distance (a and d), latency to find the platform (b and e), and swim speed (c and f). Data are presented as means ±S.E.M. $^{\#}P<0.05$ vs. control group; $^{*}P<0.05$ vs. model group. $n = 10$.

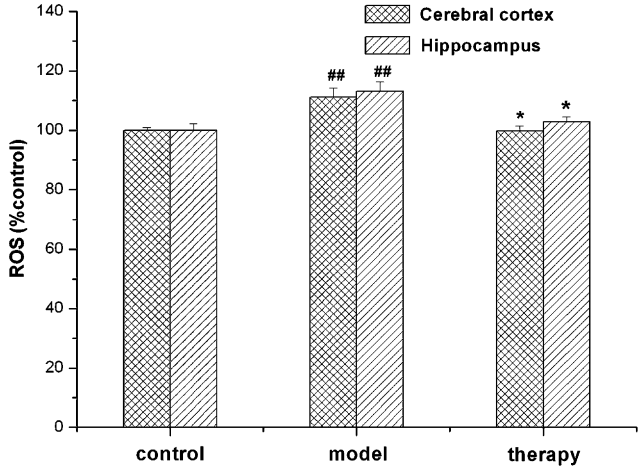

Fig. 3. Effects of LPS and catalpol on generation of reactive oxygen species in mice cerebral cortex and hippocampus mitochondria evaluated 4 h after saline vehicle or LPS administration ($n = 10$). The results are expressed as percentage of control and presented as mean ± S.E.M. $^{\#\#}P< 0.01$ vs. control group; $^{*}P<0.05$ vs. model group.

and catalpol may decrease the release of ROS to protect mitochondrial function induced by rotenone (Mao et al., 2007).

The activity of MPTP opening

Opening of the MPTP is important in mitochondrial events leading to programmed cell death. The activity of MPTP opening was measured as described in the section on Measurement of MPTP opening with calcein AM. As compared to the control group mice, the activity of MPTP in cerebral cortex and hippocampus significantly increased in the model group mice ($P<0.05$ vs. control group) and catalpol (10 mg/kg d) could decline the activity in cerebral cortex($P<0.05$ vs. model group) and hippocampus ($P<0.01$ vs. model group) as shown in Fig. 4.

Mitochondrial membrane potential

The loss of mitochondrial membrane potential is the result of the opening of permeability transition pores, or mega channels. The mitochondrial membrane potential was measured as described in the Measurement of mitochondrial membrane potential section. As shown in Fig. 5, LPS (100 μg/mouse, i.p.) induced a significant loss of mitochondrial membrane potential in model group in cerebral cortex ($P<0.05$) and hippocampus

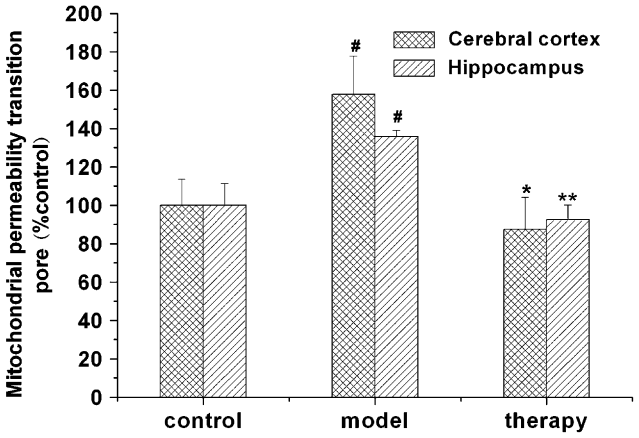

Fig. 4. Effects of LPS and catalpol on degree of MPTP opening in mice cerebral cortex and hippocampus evaluated 4 h after saline vehicle or LPS administration ($n = 10$). The results are expressed as percentage of control and presented as mean ± S.E.M. $^{\#}P <$ 0.05 vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

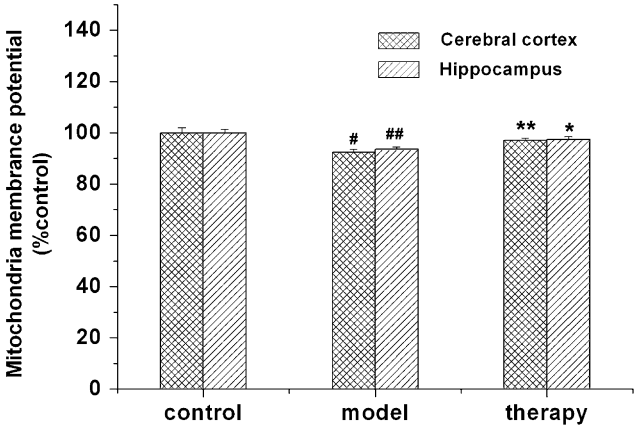

Fig. 5. Effects of LPS and catalpol on mitochondrial membrane potential in mice cerebral cortex and hippocampus mitochondria evaluated 4 h after saline vehicle or LPS administration ($n = 10$). The results are expressed as percentage of control and presented as mean ± S.E.M. $^{\#}P <$ 0.05, $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

mitochondria ($P < 0.01$), but catalpol (10 mg/kg d, i.p.) pretreatment rescued markedly the loss of mitochondrial membrane potential in the therapy group (cerebral cortex, $P < 0.01$; hippocampus, $P < 0.05$).

Effects of LPS and catalpol on NF-κB activity

NF-κB transcription factors are present in the cytoplasm in an inactive state, complexed with inhibitory IκB proteins, which cover the nuclear localization structures of NF-κB. Expression of NF-κB p65 in nucleus reflects the activation of NF-κB. The percentage of positive NF-κB p65 cells in the control group was 0.5% and 0.7% in cerebral cortex and hippocampus, respectively (Fig. 6A). After treatment with LPS (100 μg/mouse) for 4 h, the percentages of positive NF-κB p65 cells were 8.2% (cerebral cortex) and 5.0% (hippocampus; Fig. 6B). However, on pretreatment with catalpol (10 mg/kg d), the percentages of positive NF-κB p65 cells declined to 0.7% (cerebral cortex) and 0.7% (hippocampus; Fig. 6C).

Discussion

Inflammation within the brain is thought to play a pivotal role on the etiology and pathogenesis of AD (McGeer and McGeer, 2001; Shibata et al., 2002). Studies suggest that peripheral infection/inflammation might affect the inflammatory state of the central nervous system (Angela et al., 2007). LPS, a product of the cell wall of gram-negative bacteria, is the active fragment of endotoxin. LPS has been found to induce infection-like sickness symptoms in experimental animals as well as humans (Kozak et al., 1994; Avistur et al., 1997). Therefore, systemically treated mice with LPS could be considered as a valid model for AD pathogenesis in its latent phase. In this study, mice were challenged with a single dose of LPS to mimic an acute peripheral infection. The purpose is to evaluate whether catalpol could mitigate LPS-induced inflammatory damage in mice.

Recio et al. (1994) have shown that catalpol possesses anti-inflammatory property. Previous studies from our laboratory have also suggested that catalpol in vitro significantly reduced the release of ROS, TNF-α, NO, and iNOS expression after LPS-induced microglial activation (Tian et al., 2006) and after $A\beta_{1-42}$-induced astrocyte activation (Jiang et al., 2008), suggesting that catalpol exerts its anti-inflammatory property by reducing the production of pro-inflammatory factors. In the present study, measurement of body weight changes and spleen indices of mice indicated that LPS exerted inflammatory damage effects. Mice injected with LPS significantly lost body weight and possessed much higher spleen indices than the control group mice. Catalpol-pretreatment mice showed no significant decrease in body weight and spleen indices were significantly lower than those of the model group, also suggesting that catalpol may exert anti-inflammatory activity.

A working memory version of the water maze required animals to effectively integrate the new information into an existing spatial schema to complete a task. Several studies reported that LPS might affect behavior in a variety of learning tasks, including the Morris water maze, though the nature of these effects varies with testing parameters (Nathan et al., 2005;

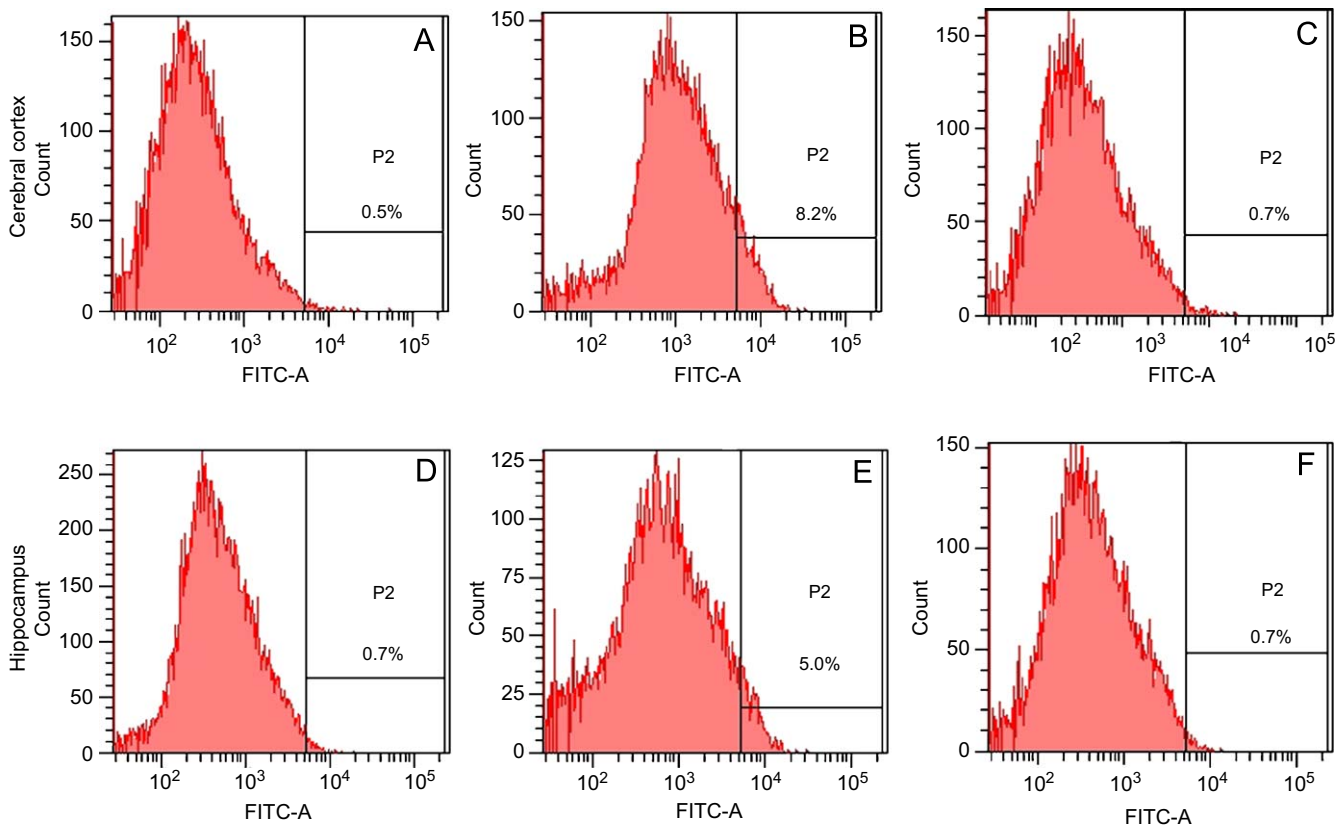

Fig. 6. Effects of LPS and catalpol on NF-κB activity in mouse cerebral cortex and hippocampus evaluated 4 h after saline vehicle or LPS administration ($n = 10$) by flow cytometric analysis. (A) control group, (B) model group, and (C) therapy group.

Chen et al., 2008). The present study demonstrated that injection with LPS impaired performance of mice in a water maze task and the therapy group mice showed a shorter latency and distance to find the hidden platform, indicating that catalpol had potential effects to prevent this kind of memory deficits induced by LPS.

In recent years, it has become increasingly clear that mitochondrial dysfunction and oxidative damage are major contributors to neuronal loss. Free radicals, typically generated from mitochondrial respiration, cause oxidative damage of nucleic acids, lipids, carbohydrates, and proteins. Emerging data from a number of neurodegenerative diseases suggest that there may be common features of toxicity that are related to oxidative damage and mitochondrial dysfunction (Orth and Schapira, 2002; Simon et al., 2008). Deficient mitochondrial metabolism may generate ROS that wreak havoc in neurons, for which mitochondrial dysfunction is such an attractive candidate for an "executioner's" role in neuronal degeneration. On the other hand, oxidative damage probably contributes to the mitochondrial dysfunction. In the present study, we found that LPS induced a loss in mitochondrial integrity as shown by a significant decrease in membrane mitochondrial potential and a significant increase in MPTP opening. This finding is consistent with previous observations of brain inflammation caused by systemic administration of LPS (Florence et al., 2007). We also observed that LPS exposure significantly increased ROS production in brain mitochondria. However, pretreatment with catalpol significantly decreased ROS production and protected mitochondrial integrity by a significant increase in membrane mitochondrial potential and decrease in MPTP opening. Potential mechanisms of neurotoxicity due to mitochondrial dysfunction are production of superoxide radical anions and enhanced oxidative stress, mitochondrial swelling and loss of energy supply, and ROS formation by opening the mitochondrial transition pore (Cassarino et al., 1999). Recently the loss of the mitochondrial membrane potential, and a large increase in mitochondrial free radical generation have been identified as the first step in the apoptotic process (Zamzami et al., 1995).

The important finding in the current study is that peripherally administered catalpol attenuated NF-κB activation in cerebral cortex and hippocampus after peripheral injection of LPS. NF-κB is a transcription factor that is involved in the regulation of the inflammatory response and coagulation by increasing the expression of numerous pro-inflammatory factors (Michael and Florian, 2005). Activation of NF-κB by the classical pathway involves phosphorylation of the

inhibitory subunit (IκBα) at serines 32 and 36, which causes its dissociation from p65-RelA/p50 followed by polyubiquination and degradation in the 26S proteasome. The antioxidant-facilitated recovery from memory deficits is due, in part, to attenuated NF-κB activity, which may be a result of decreased ROS in brain. A reduction in ROS corresponds to decreased NF-κB activity in a variety of immune cells and animal experimental studies (Haddad, 2002; Godbout et al., 2005b). In this study, we showed that pretreatment with catalpol attenuated NF-κB activation induced by LPS, using flow cytometry. The structure of catalpol was very similar to those of aucubin and catalposide, which have been reported to be anti-inflammatory (An et al., 2002). The structures of hydroxylation and unsaturation are related to anti-inflammatory activity. It is concluded that the effects of catalpol may be achieved by maintaining mitochondrial function via decreasing ROS production and attenuating NF-κB activation.

In conclusion, our findings indicate that peripheral administration of LPS causes memory impairment and mitochondrial dysfunction in mice cerebral cortex and hippocampus, including decrease in membrane mitochondrial potential and increase of MPTP opening and ROS production, which also induces NF-κB activation. Catalpol pretreatment significantly improves the cognitive impairment, maintains the mitochondrial integrity, and attenuates NF-κB activation in the brain of mice. Therefore, catalpol may have potential neuroprotective effect in LPS-induced acute systemic inflammation.

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