BRAIN RESEARCH 1188 (2008) 139–147

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Research Report

Catalpol attenuates the neurotoxicity induced by β-amyloid$_{1–42}$ in cortical neuron–glia cultures

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ARTICLE INFO

Article history:
Accepted 18 July 2007
Available online 22 October 2007

Keywords:
Catalpol
Aβ$_{1–42}$
Glia
Neuron
Inflammatory

ABSTRACT

A glia-mediated inflammation plays an important role in the pathogenesis of Alzheimer's disease (AD). In vitro, besides a direct neurotoxic effect on neurons, Aβ activates glia to produce an array of inflammatory factors including tumor necrosis factor-α (TNF-α), reactive oxygen species (ROS), nitric oxide (NO) and inducible nitric oxide synthase (iNOS), which accelerate the progression of AD. Catalpol, an iridoid glycoside, isolated from the root of *Rehmannia glutinosa*, protects neuronal cells from damage caused by a variety of toxic stimulus. In the present study, the effect of catalpol against Aβ$_{1–42}$-induced neurotoxicity in primary cortical neuron–glia cultures as well as its mechanism acting on cells was further investigated. Pretreatment with catalpol at the dosage of 500 μM for 30 min prior to 5 μM Aβ$_{1–42}$ not only attenuated the Aβ$_{1–42}$-triggered neurotoxicity to neurons but also inhibited the glial activation to some extent, which was examined by inspecting the morphological changes and measuring the release of the above mentioned inflammatory factors. Therefore, the results demonstrated that catalpol might be a promising anti-inflammatory agent in the therapy or prevention of neurodegenerative diseases associated with inflammation.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease characterized by progressive memory loss, cognitive decline and widespread loss of neurons and their synapses in the cerebral cortex, entorhinal area, hippocampus, ventral striatum and basal forebrain. Brains of individuals with AD manifest two characteristic lesions: extracellular amyloid (or senile plaques) and intracellular neurofibrillary tangles of hyperphosphorylated tau protein (Selkoe, 2001). Immunocytochemistry has been revealed that senile plaques are associated with activated astrocytes and abundant microglia at the site of deposition of Aβ in AD (Bernhardi and Eugenin, 2004) and the activation of the inflammatory response is considered to play a key role in determining the rate of the neurodegenerative process (McGeer and McGeer, 1998). β-Amyloid protein (Aβ), a major component of senile plaques, which is most abundant in the cortex of AD patients, has toxic effects on neurons and induces the activation of microglia and astrocytes in vitro (Malchiodi-Albedi et al., 2001; Rogers et al., 2002).

It seems that the glial cells are to be responsible for the neuroinflammation observed in AD. Activated microglia and reactive astrocytes surrounding extracellular deposits of Aβ initiate an inflammatory response characterized by a local cytokine-mediated acute phase response, activation of the complement cascade and subsequent further cell damage (Aisen, 1997; McGeer et al., 1993). Microglia consist around 10% of the cells in neural system. They represent the first line of

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doi:10.1016/j.brainres.2007.07.105

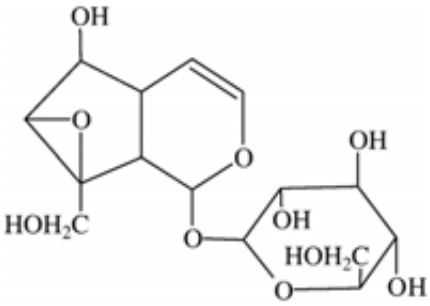


Fig. 1 – The chemical structure of catalpol. Catalpol, an effective and active ingredient, isolated from the Chinese traditional herbs *Rehmannia glutinosa*, is an iridiod glucoside, with a small molecular weight of 362.45.

defense against invading pathogens or other types of brain tissue injury (Sastre et al., 2006). However, uncontrolled over-activation of the microglia can be detrimental, leading to further damage and exacerbating disease processes. Activated microglia release a variety of proinflammatory cytokines, NO, ROS and TNF-α, all of which are toxic to neuronal cells. While TNF-α is the major neurotoxic agent secreted by Aβ-stimulated microglia (Combs et al., 2001). Astrocytes are the major glial cells present in CNS. In counting, they greatly outnumber neurons, microglia or oligodendrocytes (Saha and Pahan, 2006). Astrocytes are not fully regarded as only passive glue-like supporting and feeding cells. They are considered as important neuronal partners influencing neural fate in neurodegeneration (Lemke, 2001), regulating neuronal survival (either by trophic or toxic mechanisms) (Malchiodi-Albedi et al., 2001) and even communicating with neurons, becoming the so-called third synaptic element (Carmignoto, 2000). As a source of immunologically relevant cytokines and chemokines, astrocytes play a pivotal role in the type and extent of CNS immune and inflammatory responses. They contribute to AD, since Aβ-activated astrocytes overexpress factors such as IL-1β, nitric oxide (NO) and S100B, which are all potentially neurotoxic to neurons (Karuppagounder et al., 2007). NO and iNOS as inflammatory molecules cause further injury to neurons and a further increase in IL-1β, a cycle of self-amplification (Griffin et al., 1998; Lee et al., 1993a,b).

In recent years, many kinds of traditional Chinese herbal medicines have been used for the therapy of AD such as HuperzineA (Zhang and Tang, 2006) and genistein (Zeng et al., 2004). Catalpol, an iridiod glucoside, isolated from the root of *Rehmannia glutinosa*, its chemical structure shown in Fig. 1, possesses a wide range of biological and pharmacological activity including purgative, anti-tumor, anti-inflammation, analgesic, sedative and anti-apoptosis properties. Although the anti-inflammatory effect of catalpol against the LPS-induced neurotoxicity in mesencephalic neuronal cells has been known (Tian et al., 2006), its molecular basis in neural system is still required to be further understood. Therefore, in the current study, to evaluate the protective effect of catalpol on cortical neuron–glia cultures induced by $A\beta_{1-42}$ and to further explore its possible mechanisms acting on cortical neurons, microglia and astrocytes in AD become the main object of this paper.

2. Results

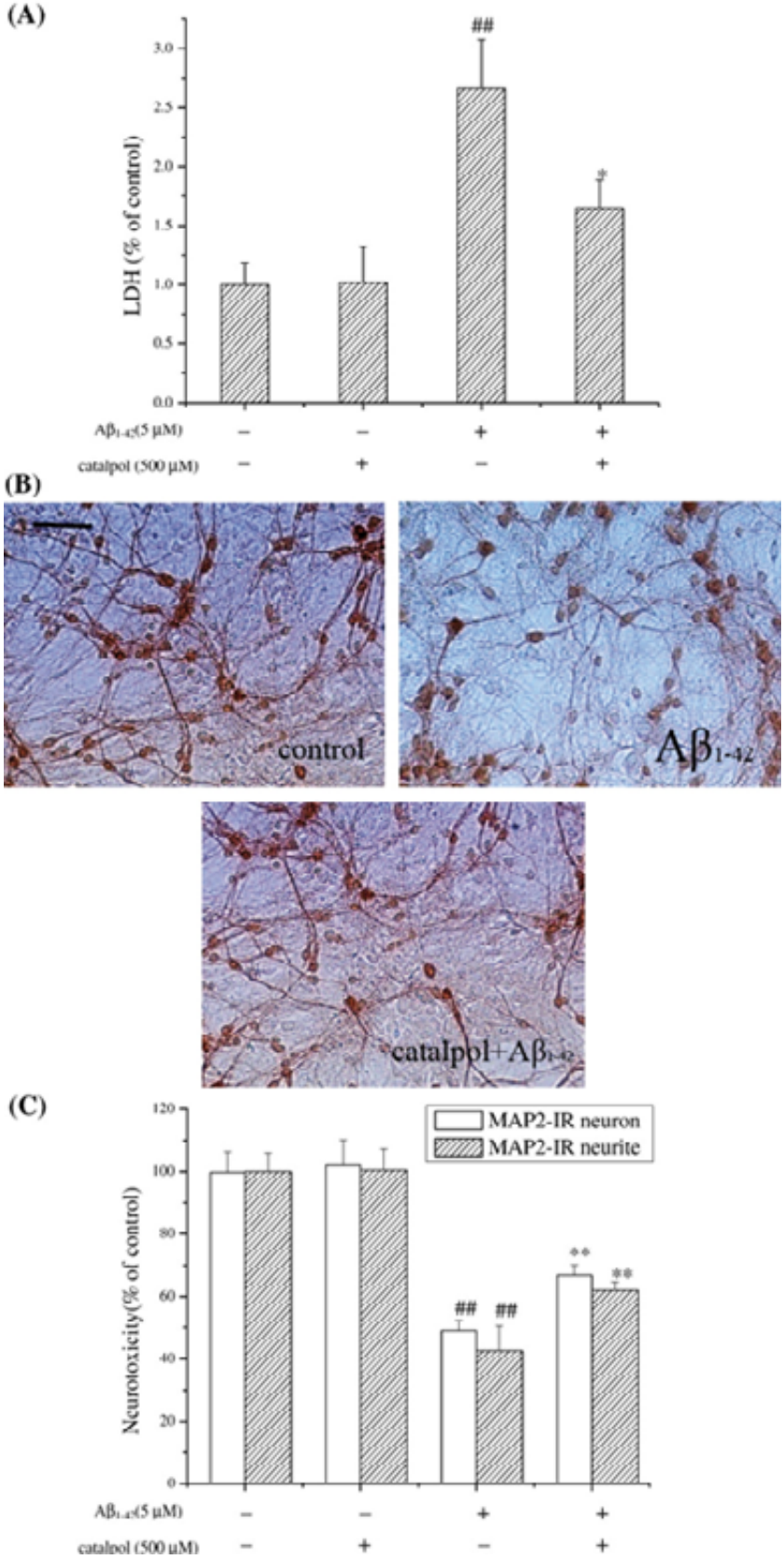
2.1. *Catalpol protects cortical neurons against $A\beta_{1-42}$-induced neurotoxicity*

In previous studies, catalpol at 500 μM was approved to be the most effective dosage to protect primary mesencephalic neurons against LPS and MPP^+-induced neurotoxicity (Tian et al., 2006; Tian and Jiang, 2007). Therefore, the involvement of catalpol at 500 μM in protecting primary cortical neuron–glia cultures from $A\beta_{1-42}$-induced neurotoxicity was further investigated in our current study. Cell cultures were pretreated with 500 μM catalpol for 30 min prior to the 5 μM $A\beta_{1-42}$-treatment for 72 h. After treatment, the culture supernatant was collected for the subsequent biochemical assays. Cell injury was quantitatively determined by measuring the generation of intracellular lactate dehydrogenase (LDH) whose release reflected a loss of membrane integrity in dying cells. In $A\beta_{1-42}$-induced cultures, the activity of LDH enhanced to 265.9±41.6% compared with control. However, pretreatment with catalpol attenuated the leakage of LDH, causing it to sharply reduce to 165.1±23.3% of control (Fig. 2A), which demonstrated that catalpol facilitated the improvement of neuronal cell viability.

Meanwhile, deterioration of cortical neurons was determined by counting the number of neuronal loss and measuring their dendrite length following immunochemical staining through the Image-Pro Plus (IPP) software. Morphologically, the changes of cortical neurons were shown in Fig. 2B, cultures treated with $A\beta_{1-42}$ for 72 h exhibited a 49.0±3.4% decrease in the number of MAP-2-positive neurons and their dendrites averagely shrank to 42.6±7.7% compared with control (Fig. 2C), showing markedly retractile and tortuous appearances as well as the neural network formed by dendrites collapsed. However,

Fig. 2 – Effect of catalpol against the $A\beta_{1-42}$-induced neurotoxicity to cortical neuron–glia cultures. (A) Cultures were treated with vehicle alone, 500 μM catalpol alone and 5 μM $A\beta_{1-42}$ alone, or pretreated with 500 μM catalpol for 30 min prior to 5 μM $A\beta_{1-42}$ for 72 h. After treatment, the release of intracellular LDH was measured to determine the $A\beta_{1-42}$-triggered cytotoxicity. However, pretreatment with catalpol reversed the generation of LDH. (B) Effect of catalpol on $A\beta_{1-42}$-induced morphological alternations in cortical neurons. The cortical neurons were immunostained with MAP-2 primary antibody as described in Experimental procedures. (C) After cell treatment, the number of MAP-2-positive cortical neurons was counted and the length of their dendrites was measured through IPP software. Pretreatment with catalpol attenuated the neurotoxicity induced by $A\beta_{1-42}$ and inhibited the degeneration of neurons to some extent. Data are expressed as means±S.E.M. of three independent experiments. $^{*}p<0.05$, $^{**}p<0.005$ compared with cultures exposure to $A\beta_{1-42}$ (5 μM) alone; $^{\#\#}p<0.005$ compared with control group. Scale bar: 50 μm.

pretreatment with catalpol (500 μM) protected the damaged neurons: not only the amount of MAP2-positive neurons increased to 66.7±3.4% but also the degeneration of their dendrites was partially counteracted, which were indicative of the neuroprotective effects of catalpol on neurons in morphology.



2.2. *Catalpol suppresses the activation of microglia triggered by $A\beta_{1-42}$*

Since inflammatory changes are overall observed in AD brain, particularly at the amyloid deposits, which are rich in active microglia (Heneka and O'banion, 2007) and it has been proposed that neuronal damage in Alzheimer's disease is not caused by accumulation of Aβ but preferentially by the inflammatory response of microglia against Aβ (McGeer and McGeer, 1998), the effects of catalpol on $A\beta_{1-42}$-induced microglial activation were required to be evaluated in microglia-enriched cultures. Under the conditions of $A\beta_{1-42}$ inducement, microglia experienced dramatic morphological changes. In control group, resting microglia were characterized by a single and regular shape, showing thin and claviform appearances. Comparing with the control samples, activated microglia presented multiform and irregular shapes, more processes extended from the cell bodies to turn into polygonal cells, as delineated in Fig. 3A. Meanwhile,

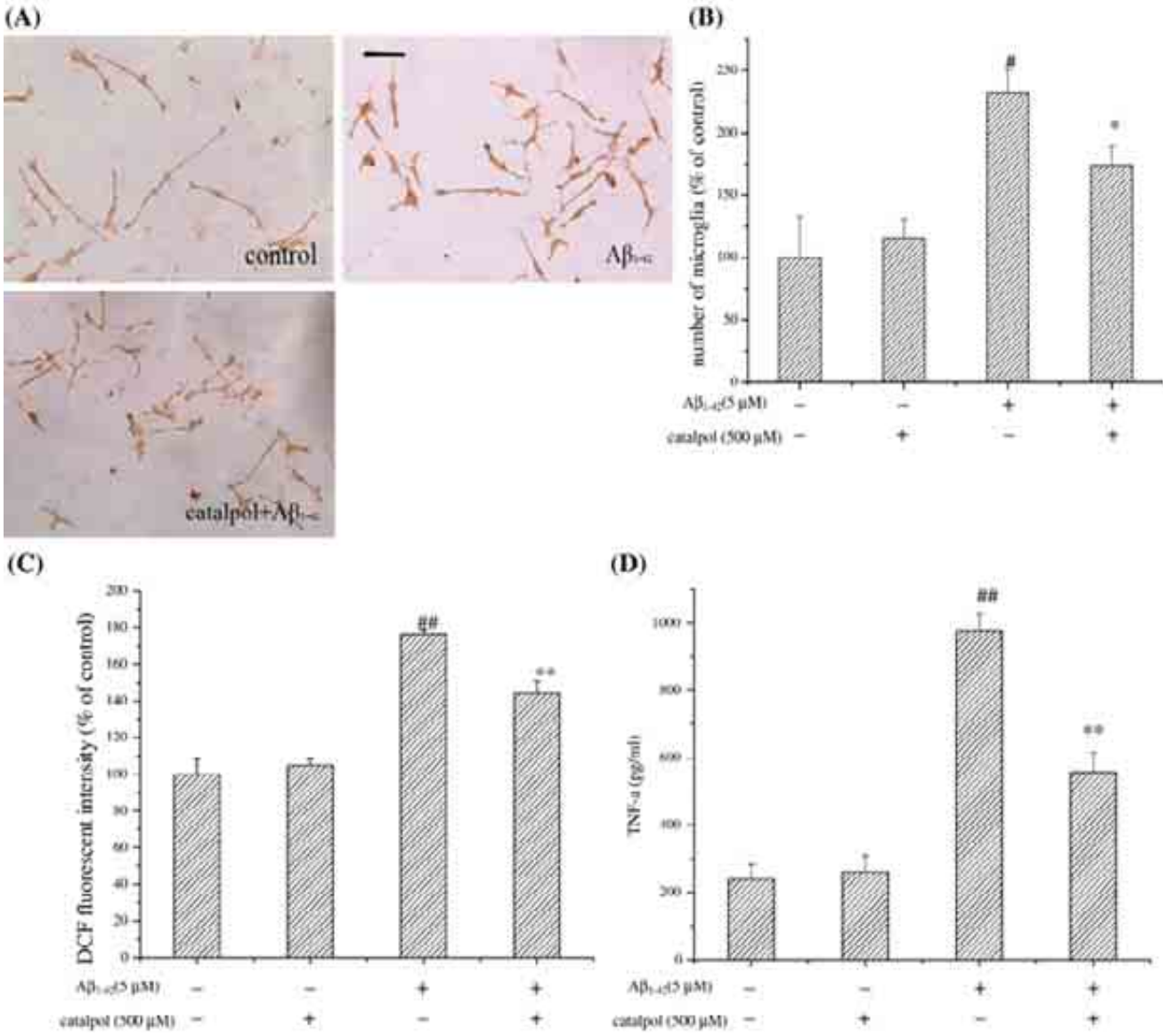


Fig. 3 – Catalpol protected microglia from the $A\beta_{1-42}$-induced activation in microglia-enriched cultures. (A) Microglia, planted on 24-well plates after 2–3 days, were treated with $A\beta_{1-42}$ (5 μM) and/or catalpol (500 μM) for 72 h. After treatment, the effect of catalpol against the microglial activation stimulated by $A\beta_{1-42}$ from morphology was inspected and cells were immunostained with CD11b primary antibody as described in the Experimental procedures. (B) After $A\beta_{1-42}$ stimulation, microglia proliferated and the number of them was counted by the Image-Pro Plus software. (C) The overexpression of ROS was fluorometrically monitored by measuring the fluorescence intensity of DCF. Pretreatment with 500 μM catalpol attenuated the accumulation of ROS induced by $A\beta_{1-42}$ in cultures. (D) In microglia-enriched cultures, the release of TNF-α was detected with ELISA kits. After treatment, catalpol inhibited the $A\beta_{1-42}$-induced excessive production of TNF-α. Data are shown as means ± S.E.M. of three independent experiments. $^{**}p<0.005$, $^{*}p<0.05$ compared with the 5 μM $A\beta_{1-42}$-treated cultures; $^{\#\#}p<0.005$, $^{\#}p<0.05$ compared with control samples. Scale bar: 100 μm.

the quantification of CD11b-positive microglia was automatically counted by Image-Pro Plus software and was found to sharply increased to 232.0±18.3% of control after $A\beta_{1-42}$ activation. After the pretreatment with catalpol, microglial activation was attenuated and the number of microglia was decreased (Fig. 3B). Microglial activation was accompanied by the generation of inflammatory factors TNF-α and ROS. After treatment with 5 μM $A\beta_{1-42}$, the production of intracellular ROS was determined by measuring the fluorescence intensity of DCF, showing a 176.6±2.3% increase, and pretreatment with catalpol (500 μM) resulted in a significant reduction to 144.2±6.6% of control and the generation of TNF-α was effectively inhibited by catalpol, as shown in Figs. 3C and D, respectively. Among the inflammatory factors secreted from activated microglia, excessive ROS and TNF-α both played prominent roles in neurodegeneration and had multiple effects in neurodegenerative disease, ultimately damaging neurons. Catalpol effectively inhibited the generation of the inflammatory factors ROS as well as TNF-α and attenuated the degree of microglial activation, which in turn protected cortical neurons from the $A\beta_{1-42}$-induced neurotoxicity.

2.3. *Catalpol blocks $A\beta_{1-42}$-induced activation to astrocytes*

Astrocytes, a source of immunologically relevant cytokines and chemokines, play a pivotal role in the type and extent of CNS immune and inflammatory responses. Recruited astrocytes, assembling at the Aβ plaque sites, most likely prolong neuroinflammation and contribute to NO-mediated neurotoxicity by expressing the iNOS and the L-arginine-supplying enzyme argininosuccinate synthesis (Karuppagounder et al., 2007). After $A\beta_{1-42}$ activation, the morphological changes in astrocytes seemed to be so tiny that it was difficult to recognize the characteristics of hypertrophy and proliferation in $A\beta_{1-42}$-activated astrocyte cultures (the figures were not shown), which presumably was associated with the innate nature of astrocytes and the concentration of $A\beta_{1-42}$ in cultures. However, the release of NO and iNOS from the activated astrocytes was detected. After $A\beta_{1-42}$ treatment for 72 h, the production of NO and iNOS both had an evident enhancement comparing with control. Pretreatment with catalpol reduced the release of NO and iNOS, as shown in Fig. 4. Catalpol effectively inhibited the activation of astrocytes to some extent, at least in part, through blocking the generation of the associated inflammatory factors NO and iNOS.

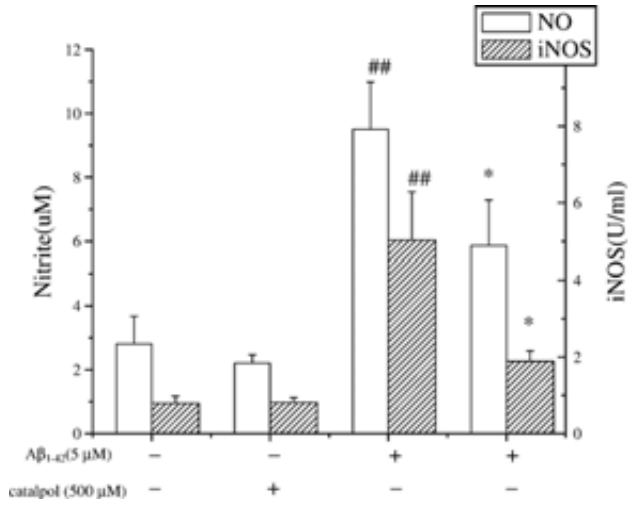


Fig. 4 – Effect of catalpol against the astrocyte activation triggered by $A\beta_{1-42}$. The astrocyte-enriched cultures planted on culture plates for 2–3 days later were pretreatment with 500 μM catalpol for 30 min prior to 5 μM $A\beta_{1-42}$ for 72 h. After cell treatment, the production of NO and iNOS released in culture medium was colorimetrically determined, respectively. Catalpol reduced the overproduction of NO and suppressed the increasing activity of iNOS. Each data is the means±S.E.M. of three independent trails. $^{*}p<0.05$ compared with $A\beta_{1-42}$-treated cultures; $^{\#\#}p<0.005$ relative to the control group.

3. Discussion

In recent years, to clarify the connection between the neurodegenerative diseases and the inflammation process has been turned into a target of experimental scrutiny. Chronic inflammation is self-propagating as neuroinflammatory molecules released by activated glia induce recruitment and activation of additional glia secreting neuroinflammatory molecules, causing further cell damage that results in additional glial activation (Griffin et al., 1998; Lee et al., 1993a,b). For this, in the present study to specially introduce the microglia-enriched cultures and the astrocyte-enriched cultures alone to further clarify the inflammatory mechanisms acting on cortical neurons is essential and necessary.

First of all, in the cortical neuron–glia cultures, exposure to $A\beta_{1-42}$ caused a direct injury to cortical neurons, which were observed through immunocytochemical staining. The number of MAP-2-positive neurons reduced and their dendrites shrank, inevitably overthrowing the intact structures of the neural network. It was indicative that the direct neurotoxicity to cortical neurons triggered by $A\beta_{1-42}$ was one of the central factors causing deterioration of cortical neurons and in turn contributed to the pathogenesis of neurodegeneration. Again, the subsequent assay of the intracellular LDH revealed that cells in cultures including cortical neurons, microglia and astrocytes were partially destroyed to death by the $A\beta_{1-42}$-induced neurotoxicity.

Since microglial activation has been associated with neurodegeneration induced by β-amyloid peptides, LPS, the human immunodeficiency virus-1 coat protein gp120 and the pesticide rotenone (Dawson et al., 1994; Liu et al., 2002; Gao et al., 2002; Qin et al., 2002) in neuron–glia cultures and microglia recently have been suggested to be preferentially associated with certain amyloid plaque types (D' Andrea et al., 2004), we explored inflammatory mechanisms acting on cortical neurons and firstly focused on microglia. It is generally recognized that activated microglia change their configuration and release inflammatory cytokines that activate and recruit other leukocytes to the brain lesion areas, resulting in neuronal damage. In microglia-enriched cultures, not only the changes in microglial morphology were inspected but also the increasing number of microglia was observed. The increasing recruitment of microglia that were synchronously neighboring neurons might encapsulate the bodies and dendrites of neurons, which directly disturbed the formation

of the intact dendrite network. Due to the integrity of the synaptic network playing a crucial role in neurotransmission, the abnormality of morphology and the outnumbering in microglia necessarily influenced the inherent nature of the CNS, leading to further dysfunction of the cortical neurons. Furthermore, the production of ROS and TNF-α released from activated microglia was found to be increasing after $A\beta_{1-42}$ stimulation in agreement with previous studies. Amyloid-dependent activation of microglia in vitro results in acquisition of a reactive phenotype with the production and secretion of proinflammatory products such as reactive oxygen species, cytokines and neurotoxins. Among the factors, ROS played a prominent role in neurodegeneration and had multiple roles in neurodegenerative disease. ROS, including superoxide anion, hydroxyl radicals and hydrogen peroxide, generated from activated microglia, is triggered by Aβ via the activation of NADPH oxidase in microglia and functions as an intracellular signaling molecule to further mediate the Aβ-induced production of inflammatory factors such as TNF-α, or NO, which in turn damage neurons (Qin et al., 2002; Kang et al., 2001; Combs et al., 2001). TNF-α, an inflammatory cytokine elevated in $A\beta_{1-42}$-activated microglia, can also cause neuronal cell death both directly and indirectly via the induction of NO and free radicals in glial cells. And, activated microglia facilitates TNF-α-mediated motor neuron death in vitro (He et al., 2002). Given all what has been mentioned above, the effective control of microglial activation in neurodegenerative diseases is surely regarded as an important therapeutic target.

In many previous studies, it has been suggested that astrocytes play a neuroprotective role of structural and trophic support to the CNS (Kirchhoff et al., 2001) and owned an antioxidant defense mechanism because they contained superoxide dismutase (SOD), glutathione peroxidase, glutathione and vitamins C and E (Wilson, 1997). However, when astrocytes activated, their detrimental effect on the CNS is not negligible at all. There were studies which demonstrated that astrocytes throughout the entorhinal cortex of AD patients gradually accumulated $A\beta_{1-42}$-positive material and that the amount of this material correlated positively with the extent of local AD pathology. $A\beta_{1-42}$-positive material within astrocytes appeared to be of neuronal origin, most likely accumulated via phagocytosis of local degenerated dendrites and synapses, especially in the cortical molecular layer (Nagele et al., 2003). Furthermore, a recent report suggested that astrocytes could be also a source for Aβ, because they overexpress BACE1 in response to chronic stress (Rossner et al., 2005). Recently, the in vitro study performed by Jill and Arlene also revealed that oligomeric and fibrillar $A\beta_{1-42}$ induced astrocyte activation and they measured IL-1β, NO, iNOS and TNF-α level as indicators of inflammation (Jill et al., 2005). Interestingly, both NO and iNOS are generated not only from the activated microglia but also from astrocytes, which exhibit neurotoxic properties and play an essential role in nerve cell death associated with the inflammatory response (Dawson et al., 1994). In the present study, the excessive expression of NO and iNOS in astrocyte-enriched cultures stimulated by $A\beta_{1-42}$ was detected, which caused great menace to survival of cortical neurons. As far as the morphological changes of astrocyte are concerned, it failed to clearly catch the characteristics of hypertrophy and proliferation as delineated in the previous studies, which was possibly associated with its innate nature. In normal conditions, astrocytes exhibited multiple shapes and extended into pieces to grow. When activated, the intersectant appearances formed by astrocytes altered too less to observe the morphological changes of a single cell. Although we failed to make a good explanation to the actions of astrocytes in morphology, the overproduction of NO and iNOS was sufficient to account for the roles of astrocytes played in the inflammatory process. The protective effects of catalpol on astrocytes alone were investigated for the first time, so more studies are needed to be performed.

The neuroprotective effects of catalpol were evaluated in the three individual culture systems, which were cortical neuron–glia cultures, microglia-enriched cultures and astrocyte-enriched cultures, respectively. In microglia-enriched cultures and astrocyte-enriched cultures, catalpol effectively attenuated the $A\beta_{1-42}$-induced neurotoxicity through inhibiting the glial activation and reducing the generation of inflammatory factors including ROS, TNF-α, NO and iNOS. In cortical neuron–glia cultures, catalpol dramatically protected cortical neurons from $A\beta_{1-42}$-triggered damage mainly via two pathways: on the one hand, catalpol attenuated the direct neurotoxicity stimulated by $A\beta_{1-42}$ to cortical neurons. On the other hand, the effective inhibition of glial inflammation by catalpol likewise rescued the cortical neurons from $A\beta_{1-42}$-triggered neurotoxicity. This was corporately reflected on the recovery of cortical neurons in morphology and the reduction of intracellular LDH release.

Taken together, catalpol, an ingredient isolated from the traditional Chinese herb *R. glutinosa*, exerted a neuroprotective effect on $A\beta_{1-42}$-triggered neurotoxicity in cortical neuron–glia cultures. Furthermore, when cultures were incubated with catalpol alone, both their morphology and the production of the related inflammatory factors had hardly any changes, which presumably accounted for the fact that catalpol had fewer side effects at the concentration of 500 μM, at least. These evidences conformably implied that catalpol could be served as a potential preventive or therapeutic drug for neurodegeneration associated with inflammation.

4. Experimental procedures

4.1. *Materials*

$A\beta_{1-42}$, purchased from Chemicon (Recombinant, *E. coli*), was resuspended in 1% NH_4OH, aliquoted at 1 mg/ml and stocked at −20 °C, according to the manufacturer's instruction. Catalpol, purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), was diluted in phosphatic buffer solution (PBS) for treatment. The polyclonal anti-MAP-2 (H-300) antibody and the monoclonal anti-CD11b antibody were purchased from Chemicon. SABC compound kits were from Sino-American Biotechnology Company. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Beyotime Institution of Biotechnology.

4.2. *Primary cortical neuron–glia cultures*

Neuron–glia cultures were prepared from cortical tissues of embryonic day 15/16 mice as previously described (Qin et al., 2002). Briefly, cortical tissues were obtained and dissociated by

a mild mechanical trituration, then the dissociated cells were planted onto poly-D-lysine-coated (20 μg/ml) 24-well tissue culture plates at a density of 5×10^5/well in Dulbecco's modified Eagle medium/nutrient F12 (DMEM/F12) (Gibco) supplemented with 10% heated-inactivated fetal bovine serum (Gibco), 50 U/ml penicillin, 1.2 g/l sodium bicarbonate and 2 mM L-glutamine. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Seven days in vitro (DIV7), the cultures were used for experiment, which contained 60% MAP-2-IR neurons, 3% CD11b-IR microglia and GFAP-IR 37% astrocytes.

4.3. *Microglia-enriched cultures and astrocyte-enriched cultures*

Microglia-enriched cultures and astrocyte-enriched cultures were prepared from the whole cerebra of 1-day-old neonatal mice. Briefly, brain tissues were triturated after removing the meninges and blood vessels, then dissociated cells were seeded into poly-D-lysine-coated 35-cm^2 T-flasks at a density of 1×10^6. The cells reached confluence on DIV12-14, then they were detached from the flasks by mild shaking for about 6 h at 180 rpm (Tian et al., 2006), the detached microglia were planted onto poly-D-lysine-coated 24-well plates at 5×10^5/well and were served for experimental assays after 2–3 days. Astrocytes were harvested as previously described (Liu et al., 2001). In brief, after separating microglia, astrocytes remained in the T-flasks, then astrocytes were detached with 0.125% trypsin–EDTA after at least consecutive five passages, seeded at 5×10^4/well onto poly-D-lysine-coated 96-well plates. After 2–3 days (that is about DIV 20–21), cultures were employed to treatment. The purity of the microglia and astrocytes in cultures was >90% identified through immunochemical staining, respectively.

4.4. *Immunocytochemistry*

Cortical neurons were stained with an antibody against microtubule-associated protein-2 (MAP-2), a marker for the cell body and neurites, and microglia were stained with an antibody against CD11b complement protein. The procedure was performed as previously described (Liu et al., 2002). Briefly, after 4% formaldehyde-fixation for 30 min, cultures were treated with 3% hydrogen peroxide for another 30 min followed by sequential incubation with blocking solution for 30 min. Cells were incubated with primary antibody dilution (MAP-2 1/400, CD11b 1/200) for 2 h at 37 °C or overnight at 4 °C. Afterwards, biotinylated anti-rabbit/anti-mouse secondary antibody was incubated with neurons/microglia for 1 h at 37 °C, respectively. Then, avidin–biotinylated enzyme complex ABC reagents were added for additional 30 min in the cultures and color was developed with 3,3′-diaminobenzidine (DAB). When color development was appropriate, it was terminated with removing DAB and washing the cells with PBS. The images were recorded under 20×objective on an inverted microscope (OLYMPUS 1X71) connected to a charge-coupled device camera. The analysis for the quantification of MAP-2-positive neurons as well as CD11b-positive microglia and the measurement of the length of neuron dendrites were performed through the Image-Pro Plus software installed in the microscope, automatically counting the number and determining the dendrite length in three independent wells of 24-well plates from five to six representative fields per well.

4.5. *Lactate dehydrogenase (LDH) assay*

On DIV 7, cortical neuron–glia cultures in 24-well plates were pretreated with catalpol (500 μM) for 30 min prior to 5 μM $A\beta_{1-42}$ for 72 h at 37 °C. At indicated time after cell treatment, the culture supernatant was collected for the subsequent biochemical assays. The release of cytosolic enzyme lactate dehydrogenase (LDH), an indicator of cytotoxicity, reflected a loss of membrane integrity in dying cells and was determined by a colorimetric assay. Its activity was measured by using commercially available kits (Jiancheng Bioengineering), according to the manufacture's instruction. Absorbance was read at 440 nm in a spectrophotometer (JASCO, V-560). The release of LDH was calculated as the percentage of control group.

4.6. *Measurement of intracellular reactive oxygen species (ROS)*

Production of ROS in microglia-enriched cultures was fluorometrically monitored using the non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA diffuses into cells where it is oxidized in the presence of ROS into the fluorescent compound 2′,7′-dichlorofluorescein (DCFH). DCFH reacts with ROS to form the fluorescent product DCF. Briefly, microglia-enriched cultures were pretreated with 500 μM catalpol for 30 min prior to 5 μM $A\beta_{1-42}$ for 72 h. After treatment, the supernatant of microglia was removed and cells were washed with PBS. DCFH-DA was diluted in fresh DMEM/F12 at a final concentration of 10 μM and incubated with microglia for 20 min at 37 °C. After DCFH-DA treatment, the chemicals were removed and loaded microglia were washed three times with PBS. The fluorescence was read at 485 nm excitation and 530 nm emission with a fluorescence plat reader (Genios, TECAN). The increasing production of ROS was expressed as a percentage of control.

4.7. *TNF-α ELISA analysis*

In microglia-enriched cultures, TNF-α was released into the culture medium. The production of TNF-α released from activated microglia was determined with mouse TNF-α commercial enzyme linked immunosorbent assay (ELISA) kits (Jingmei Biotech Co., Ltd.), as described by the manufacturer's protocol.

4.8. *NO and iNOS assays*

In astrocyte-enriched cultures, both NO and iNOS were generated after $A\beta_{1-42}$-inducement and exhibited neurotoxic properties. The production of NO was determined by measuring the accumulation of nitrite release into the medium using a colorimetric reaction with the Griess reagent (Green et al., 1982) with serial dilution sodium nitrite dissolved in the

culture medium used as standards. Briefly, after the treatment with 5 μM $A\beta_{1-42}$ reacting with astrocytes for 72 h in the presence or absence of 500 μM catalpol prior, the culture supernatant was collected and mixed with an equal volume of Griess reagent (0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4) and developed for 10 min. The absorbance at 545 nm was read with a microplate reader (sunrise, TECAN). The expression of iNOS in culture medium was further confirmed by using commercially available kits (Jiancheng Bioengineering), performed under the instruction of manufacturer's completely. The assay for iNOS activity relied on its ability to catalyze Ariginine (Arg) to form NO, which can further react with nucleophilic substance to produce a chromophoric compound that has the largest absorbance at 530 nm.

4.9. *Statistical analysis*

The data are presented as means ± S.E.M with $p<0.05$ considered significant. Statistical significance was determined using Student's unpaired *t* test with equal variance.

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