

Catalpol protects primary cultured astrocytes from in vitro ischemia-induced damage

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Abstract

Catalpol, an iridoid glycoside abundant in the roots of *Rehmannia glutinosa*, has been previously found to prevent the loss of CA1 hippocampal neurons and to reduce working errors in gerbils after ischemia-reperfusion injury. In the present study, we investigated the effects of catalpol on astrocytes in an ischemic model to further characterize its neuroprotective mechanisms. Primary cultured astrocytes exposed to oxygen-glucose deprivation (OGD) followed by reperfusion (adding back oxygen and glucose, OGD-R), were used as an in vitro ischemic model. Treatment of the astrocytes with catalpol during ischemia-reperfusion increased astrocyte survival significantly in a concentration-dependent manner, as demonstrated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH) release and morphological observation. In addition, catalpol prevented the decrease in mitochondrial membrane potential, inhibited the formation of reactive oxygen species (ROS) and the production of nitric oxide (NO), decreased the level of lipid peroxide and the activity of inducible nitric oxide synthase (iNOS), and elevated the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and the content of glutathione (GSH). Our results suggest that catalpol exerts the most significant cytoprotective effect on astrocytes by suppressing the production of free radicals and elevating antioxidant capacity.

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Keywords: Catalpol; Astrocyte; Ischemia; ROS; iNOS

1. Introduction

Cerebral ischemia induces neural cell injury in the CNS (Lipton, 1999). Although the mechanism of neural cell death has not exactly been demonstrated in ischemia, oxidative stress is suggested to be one of the major mechanisms of ischemic cell death (Kitagawa et al., 1990; Floyd, 1999; Love, 1999). Oxidative stress refers to an imbalance between the intracellular productions of reactive oxygen species (ROS) and the cellular defense mechanisms, when an excess of ROS is accompanied by a reduced capability of the natural antioxidant systems. Under physiological conditions, basal amounts of ROS are generated, participate in many important physiological processes (Droge, 2002; Turpaev, 2002; Voeikov, 2006), and

are rapidly scavenged by endogenous antioxidant systems, including antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) and low-molecular weight antioxidants, such as glutathione (GSH). In contrast, excessive production of ROS overwhelms cellular defense systems after cerebral ischemia/reperfusion and may either directly damage cellular macromolecules, such as lipids and nucleic acids, to cause cell necrosis, or indirectly affect normal cellular signaling pathways and gene regulation to induce apoptosis (Facchinetti et al., 1998; Lievre et al., 2000; Chan, 2001; Sugawara and Chan, 2003). Therapeutic efforts aimed to remove ROS or inhibit their formation have been shown to be beneficial in ischemia (Tagami et al., 1998; Cuzzocrea et al., 2000; Yamada et al., 2003).

Astrocytes, the most abundant glial cell types in the brain, have been known to carry out a number of functions including modulation of synaptic transmission and plasticity, secretion of growth factors, uptake of neurotransmitters, regulation of

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extracellular ion concentrations and metabolic support of neurons (Muller et al., 1995; Nedergaard et al., 2003; Newman, 2003; Slezak and Pfrieger, 2003; Hertz et al., 2004). They are also a central component of the brain's antioxidant defense (Desagher et al., 1996; Dringen et al., 2000). The ability of astrocytes to maintain these functions may be a critical determinant of neuronal survival and brain function after ischemia (Rosenberg, 1991; Blanc et al., 1998). Thus, astrocytes could be one of the targets for neuroprotection (Takuma et al., 2004).

Nitric oxide (NO), a gaseous free radical (Dawson et al., 1992) and ubiquitous neurotransmitter, also plays a critical role in ischemic brain injury (Samdeni et al., 1997). It is synthesized from arginine by three isoforms of nitric oxide synthase (NOS) (Griffith and Stuehr, 1995): the constitutive calcium-dependent neuronal (nNOS) and endothelial (eNOS) isoforms, and inducible calcium-independent (iNOS) isoform. NO can be neuroprotective or neurotoxic during cerebral ischemia depending on the NOS involved (Iadecola, 1997). The production of NO by eNOS might have beneficial effects (Huang et al., 1996), including maintenance of cerebral blood flow and inhibition of platelets aggregation and polymorphonuclear neutrophil adherence, whereas NO produced by nNOS and iNOS appears to be deleterious. These deleterious effects were demonstrated by pretreating NOS knockout mice (Iadecola et al., 1997) and rats with NOS inhibitors (Mori et al., 2001), which led to a decrease in infarct volumes compared with controls. Endothelial NOS and nNOS are expressed constitutively and their activities are regulated by intracellular Ca^{2+} . NO production by nNOS and eNOS is believed to occur in small bursts only when intracellular Ca^{2+} concentration increases. In contrast, iNOS is not normally present in most cells, but is induced in astroglial and microglial cells under ischemic conditions (Nomura and Kitamura, 1993; Iadecola et al., 1995a,b). In contrast with nNOS and eNOS, iNOS is not regulated by intracellular Ca^{2+} , and produces NO continuously and in large amounts. Induction of iNOS might contribute to delayed neuronal injury (Chen et al., 1993). Thus, interventions of astrocytic production of NO in ischemic brain injury should provide new therapeutic strategies for treating ischemic injury.

Rehmannia glutinosa, an herb used in traditional Chinese medicine, has been used to treat a whole host of ailments for centuries. It is believed that the presence of iridoid glycosides in *Rehmannia*, which have a wide variety of biological activities including helping with diabetes, anemia, dizziness; stimulating the adrenal glands produce anti-inflammatory molecules and contributing to the production of sex hormones in menopausal women (Liang et al., 1999; Yokozawa et al., 2004). Catalpol an iridoid glycoside is one of the major active components in *Rehmannia* and has been chosen as a marker substance for the chemical evaluation or standardization of *Rehmannia* and its products (Xiong et al., 2003). Recently, we have found that catalpol also has antioxidative (Jiang et al., 2004) and anti-ischemic (Li et al., 2004) effects in vitro and in vivo. However, the effects of catalpol on astrocytes during ischemia remain unclear. Therefore, the purpose of our study is to investigate the

protective effects of catalpol on cultured astrocytes and elucidate its mechanisms in antioxidant systems during ischemia.

2. Experimental procedures

2.1. Materials

Catalpol was of analytical grade (purity > 98%) and was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), poly-D-lysine and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium with Ham's nutrient mixtures F12 (DMEM/F12), glucose-free DMEM and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY). 2',7'-Dichlorofluorescein diacetate was obtained from Beyotime. The polyclonal antibody against glial fibrillary acidic protein (GFAP) and avidin–biotin complex (ABC) kit were purchased from Beijing Zhongshan Company (Beijing, China). The reagents used in enzyme assays were provided by the Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Primary astrocyte cultures

Primary mixed glial cultures were prepared from newborn mouse brains as previously described by Han et al. with minor modifications (Han et al., 2000). Briefly, the Kunming mouse brains were excised aseptically and the meninges and the blood vessels were removed carefully. Brains were dissociated into a single cell suspension by mild mechanical trituration in ice-cold growth medium (DMEM/F12) containing 10% heat inactivated FBS and 50 U/ml penicillin. The cell suspension was centrifuged at 1000 rpm for 10 min at 4 °C and the pellet was resuspended in growth medium. Cells were then placed into 75 cm² tissue culture flasks precoated with poly-D-lysine (20 µg/ml). Cultures were incubated in a CO₂ incubator at 37 °C in an atmosphere of 5% CO₂ in air. The medium was changed after 1–2 days and then twice a week. On reaching confluence, the cultures were shaken for 3 h at 180 rpm to remove microglia and oligodendrocyte progenitors. The remaining astrocyte monolayers were trypsinized and replated at an approximate density (1×10^6 cell/ml) in precoated plates. Before use, astrocytes were grown to confluence. To identify astrocytes, cultures were stained via immunocytochemistry for GFAP. Analysis of the cultures has shown that 90–95% of cells were GFAP-positive. All experiments were performed on 21–30 day cultures.

2.3. In vitro model of ischemia

The in vitro model of ischemia in this experiment was used to maintain the cultures under oxygen-glucose deprivation (OGD) for 3 h, followed by 20 h of reperfusion. Briefly, on the day of the experiment, the culture media was removed and cultures were washed three times with glucose-free DMEM. The cultures plates were then placed into an anaerobic chamber, flushed with the anaerobic gas mixture (95% N₂ and 5% CO₂), and kept in the anaerobic chamber within the 37 °C incubator for 3 h. Following OGD, the culture plates were taken out from the anaerobic chamber, glucose was added to a final concentration of 4.5 mg/ml and the cultures were incubated under normal growth conditions for an additional 20 h. Different concentrations (0.3 µM, 2.8 µM, 27.6 µM and 275.9 µM) of catalpol were added to the media during OGD and the cultures were kept until the end of OGD-R. Control cultures with fetal serum-free DMEM were cultured in the incubator under normal growth conditions.

2.4. Assessment of cell survival

Astrocytic survival was determined by assaying the degree of methylthiazol tetrazolium (MTT) reduction and the amount of lactate dehydrogenase (LDH) released into the culture medium. Cells were seeded into 96-well plates and subjected to various treatments. Following treatments, 10 µl MTT solution (5 mg/ml in phosphate buffered saline, PBS) was added into each well and the

cells were further incubated for 3 h at 37 °C. Afterwards, the media were aspirated and 100 µl of dimethyl sulfoxide (DMSO) was added for the dissolution of formazan crystals. The absorbance of each well was read at 570 nm using an ELISA plate reader. Cell viability was expressed as a percentage of the control culture. After various treatments, the amount of LDH released into the medium and total cellular LDH were also determined using a diagnostic kit (Jiancheng Bioengineering Institute, Nanjing, China). In brief, NADH and pyruvate (0.1%, w/v) were added and the samples were incubated at 37 °C for 15 min. Next, the samples were incubated with the coloring reagent for 15 min. The reaction was stopped by adding 0.4 mol/l NaOH and the activity (U/ml) of LDH in each sample were calculated from formula. The total LDH release that corresponded to complete astrocyte death was determined at the end of experiments following repeated freezing and thawing. Cell death was expressed as percentage of total LDH.

2.5. Assessment of morphology

To examine the cellular and nuclear morphology of astrocytes, cells were observed by phase-contrast microscope or fluorescence microscope after staining with Hoechst 33258. Cells were seeded into 24-well plates. After various treatments, cells were observed by phase-contrast microscope, and then fixed for 15 min with 4% paraformaldehyde at room temperature. Subsequently, cells were washed twice with PBS and were stained with Hoechst 33258 (5 µg/ml in water) for 30 min at room temperature. After three washes, the cells were observed under fluorescence microscope with excitation at 360 nm.

2.6. Measurement of ROS and MDA

Reactive oxygen species were measured with the non-fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Possel et al., 1997). DCFH-DA passively diffuses into cells and is hydrolysed by cellular esterases to 2',7'-dichlorofluorescein (DCFH), a non-fluorescent molecule that can be oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of peroxides. Cells were seeded into 24-well plates and subjected to various treatments. Following treatment, cells were incubated with 10 µM DCFH-DA dissolved in cell-free medium at 37 °C for 30 min and then washed three times with PBS. Cellular fluorescence was quantified using fluorescence microplate reader (Genios, TECAN) at an excitation of 485 nm and an emission of 538 nm. Intracellular ROS production was expressed as a percentage of control cells. Malondialdehyde (MDA), a product of lipid peroxidation (LPO), was analyzed by using an assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The concentration of MDA can be measured at a wavelength of 532 nm by reacting with thiobarbituric acid (TBA) to form a stable chromophoric production. The level of MDA was expressed as nmol of MDA per milliliter.

2.7. Measurement of NO and iNOS activity

The production of NO was estimated from the accumulation of nitrite (NO₂⁻), a stable end product of NO metabolism, in the medium using the Griess reagent as described previously. Equal volumes of culture supernatant and Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) were mixed and incubated for 15 min at room temperature. The absorbance was determined at 540 nm using a microplate reader and calibrated with a nitrite standard curve to determine the nitrite concentration in supernatants. iNOS activity was measured by using an assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The assay for iNOS activity depends on the ability of the synthase to catalyze arginine (Arg) to form NO, which can further react with nucleophilic substances to produce chromophoric compound, which has a peak absorbance at 530 nm. iNOS activity can be determined based on fact that nNOS and eNOS were Ca²⁺-dependent, while iNOS was Ca²⁺-independent. One unit of iNOS activity was defined as the amount that formed 1 nmol NO in 1 min per milliliter medium.

2.8. Measurement of SOD, GPx activities and GSH

The activities of SOD, GPx and the content of GSH were all determined by using assay kits (Jiancheng Bioengineering). The assay for SOD activity was

based on its ability to inhibit the oxidation of oxyimine by O₂⁻ produced from the xanthine–xanthineoxidase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%. The assay for GPx activity was assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H₂O₂ catalyzed by GPx. One unit of GPx was defined as the amount that reduced the level of GSH by 1 µmol l⁻¹ in 1 min per milligram protein. GSH was measured based on that 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reacts with GSH to generate 2-nitro-5-thiobenzoic acid and GSSG. Since 2-nitro-5-thiobenzoic acid is yellow, the GSH concentration in a sample solution can be determined by OD measurement at 412 nm absorbance. The concentration of GSH was calculated from formula and expressed as nmol per milligram protein. Cells were plated into 6-well plates. After various treatments, cultures were washed twice in ice-cold PBS (pH 7.4) and homogenized. The homogenate was centrifuged for 10 min at 10,000 rpm at 4 °C and supernatant was used for SOD, GPx activities and GSH assays according to the manufacturer's instructions. Protein content was measured by Coomassie blue protein-binding method using bovine serum albumin as standard.

2.9. Measurement of mitochondrial membrane potential

The changes in mitochondrial membrane potential (MMP) were estimated using the fluorescent cationic dye Rhodamine 123 (Rh 123), as previously reported (Zamzami et al., 2001). In brief, cells were seeded in 24-well plates. After treatments, cells were incubated with 10 µM Rh 123 at 37 °C for 30 min and then washed twice with PBS. The cellular fluorescence intensity of Rh 123 was quantified using fluorescence microplate reader (Genios, TECAN) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Cellular mitochondrial membrane potential was expressed as a percentage of control cells.

2.10. Statistical analysis

All the experiments were performed in triplicate and data are shown as mean ± S.D. based on three separate experiments. Statistical analysis was performed according to the Student's *t*-test and one-way ANOVA analysis. The probability values of *P* < 0.05 were considered as significant.

3. Results

3.1. Effects of catalpol on the survival of cultured astrocytes

Prior to investigating effect of catalpol on astrocytic injury induced by OGD-R, an experiment was performed to determine whether catalpol was toxic to astrocytes. After 24 h of incubation in the absence (control) or presence of different concentrations (0.3 µM, 2.8 µM, 27.6 µM and 275.9 µM) of catalpol, cell viability was quantified by MTT assay. At indicated concentrations of catalpol, the viability of astrocytes did not differ from control cells (data not shown). Thus, catalpol was used at concentrations of 0.3 µM, 2.8 µM, 27.6 µM and 275.9 µM in all experiments. Exposure of astrocytes to OGD for 3 h followed by 20 h reoxygenation in vitro led to 57.5 ± 4.2% attenuation of MTT reduction. The cell viability of astrocytes treated with catalpol during OGD-R significantly increased to 47.2 ± 3.7%, 58.8 ± 1.9%, 68. ± 10.0%, and 88.3 ± 5.7% at 0.3 µM, 2.8 µM, 27.6 µM and 275.9 µM (Fig. 1A), respectively, in comparison with catalpol-untreated cells. Similar results were shown by LDH release. In cultures exposed to OGD-R alone, LDH release was 41.5 ± 3.5% of total content. However, in cultures treated with catalpol, LDH

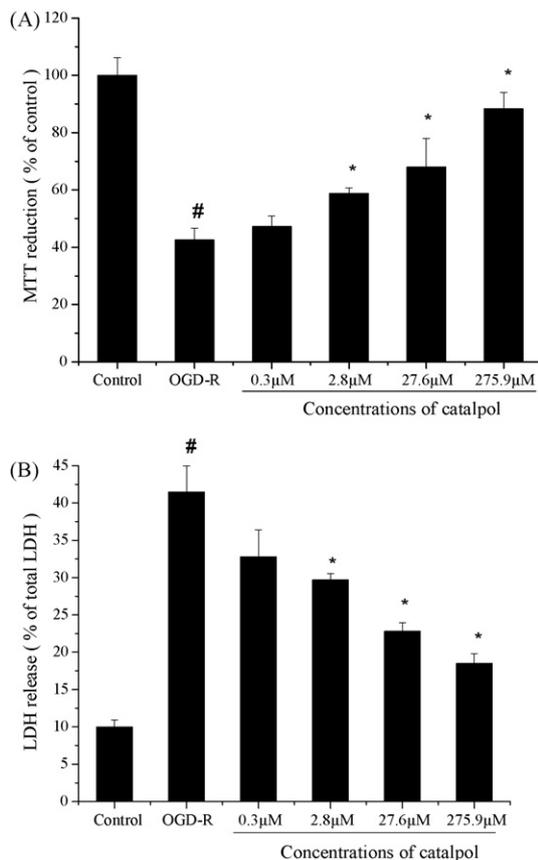


Fig. 1. Effect of catalpol on the survival of cultured mouse astrocytes. Astrocytes exposed to normal oxygen and glucose or oxygen-glucose deprivation for 3 h followed by 20 h reoxygenation in the absence or presence of various concentrations (0.3 μ M, 2.8 μ M, 27.6 μ M and 275.9 μ M) of catalpol. Cell survival was measured by MTT conversion assay (A) and LDH release (B). Data are shown as mean \pm S.D. from three separate experiments; the symbol (#) $P < 0.05$ as compared with control; the symbol (*) $P < 0.05$ as compared with OGD-R.

release decreased to $32.8 \pm 3.6\%$, $29.7 \pm 0.8\%$, $22.8 \pm 1.2\%$ and $18.5 \pm 1.3\%$ of control at 0.3 μ M, 2.8 μ M, 27.6 μ M and 275.9 μ M, respectively (Fig. 1B). These data indicate that adding catalpol prior to OGD insult could reduce cell death and elevate cell viability.

3.2. Effects of catalpol on the morphologic changes

The protective effects of catalpol were also shown through morphological observation. The OGD-R insult was accompanied by the cellular and nuclear morphological changes. Visual inspection by phase-contrast microscopy demonstrated that those normal primary cultured astrocytes were flat polygons grown in a confluent monolayer (Fig. 2A). After ischemic incubation, astrocytes displayed obvious cell body shrinkage, nuclear condensation and loss in cell membrane integrity (Fig. 2B). The addition of catalpol, however, dramatically prevented cell morphological deterioration. Most of the astrocytes showed normal cell morphology with normal nuclear size and integrity (Fig. 2C–F). The specific DNA fluorescent dye, Hoechst 33258, was used to assess the change in DNA and nuclear structure following different treatments. As

shown in Fig. 2, nuclei in untreated astrocytes were large and regular (Fig. 2a). In contrast, most of the nuclei in ischemic astrocytes were highly condensed (Fig. 2b), which is a morphological indication typically seen in apoptotic cells. In the presence of 0.3 μ M, 2.8 μ M, 27.6 μ M and 275.9 μ M catalpol during OGD-R, a significant reduction in number of the condensed nuclei in the cultures was observed (Fig. 2c–f). Most of these nuclei maintained their normal shape and size.

3.3. Effects of catalpol on ROS and MDA

To determine whether catalpol exerts its protection by inhibiting ROS production, we assayed the generation of ROS and the levels of MDA, which is a product of membrane lipid peroxidation. Astrocyte exposure to OGD-R markedly increased the generation of ROS and the content of MDA to $244.2 \pm 7.2\%$ and $362.0 \pm 24.1\%$ of control. Catalpol was able to reduce the generation of ROS induced by OGD-R. Results indicated that the levels of ROS decreased to $231.0 \pm 8.5\%$, $186.5 \pm 5.3\%$, $144.9 \pm 5.9\%$ and $103.5 \pm 4.6\%$ of control at 0.3 μ M, 2.8 μ M, 27.6 μ M and 275.9 μ M of catalpol (Fig. 3A), respectively. Moreover, the levels of MDA also significantly decreased to $241.3 \pm 20.3\%$, $175.8 \pm 3.0\%$, $117.2 \pm 23.1\%$ and $89.6 \pm 28.3\%$ by 0.3 μ M, 2.8 μ M, 27.6 μ M and 275.9 μ M catalpol (Fig. 3B).

3.4. Effects of catalpol on NO production and iNOS activity

To determine whether protective effects of catalpol against OGD-R-induced astrocytic injury via an NO-mediated mechanism, the amount of NO produced was measured. Compared to the control cells, OGD-R strongly induced the production of NO. OGD-R-induced NO production was markedly reduced by catalpol treatment (3 μ M, 2.8 μ M, 27.6 μ M and 275.9 μ M) (Fig. 4A). Results indicated that the activity of iNOS, which induces NO production, was also diminished by catalpol (Fig. 4B).

3.5. Effects of catalpol on SOD, GPx and GSH

The average GSH content was 11.9 ± 2.1 nmol/mg of protein, and the activities of SOD and GPx were 21.3 ± 4.5 U/mg and 28.4 ± 3.8 U/mg protein in the astrocyte cultures, respectively. After OGD-R, the astrocytic antioxidant system was disturbed, namely, the activities of SOD and GPx, and the content of GSH decreased to $52.0 \pm 5.6\%$, $54.5 \pm 3.1\%$ and $60.6 \pm 5.98\%$ of control (Fig. 5). Catalpol significantly preserved the activities of the antioxidant enzymes, SOD (Fig. 5A) and GPx (Fig. 5B), and prevented the depletion of GSH (Fig. 5C) under OGD-R conditions.

3.6. Effects of catalpol on mitochondrial membrane potential

Mitochondria are central to both normal cell function and the regulation of cell death. One additional effect of

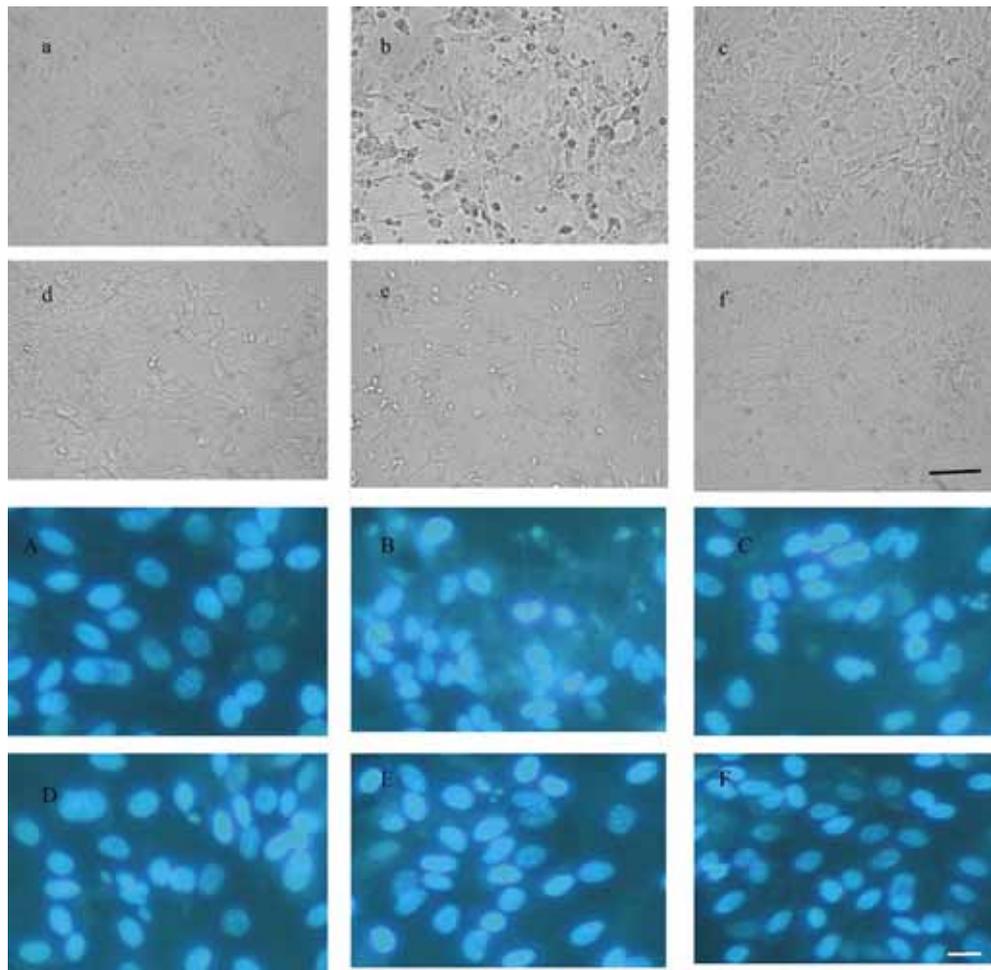


Fig. 2. Effect of catalpol on the cell morphology in cultured mouse astrocytes. Astrocytes exposed to normal oxygen and glucose (a, A), or oxygen-glucose deprivation for 3 h followed by 20 h reoxygenation in the absence (b, B) or presence of various concentrations (0.3 μM , 2.8 μM , 27.6 μM and 275.9 μM) of catalpol (c, C; d, D; e, E; f, F). Cellular morphology was observed with light microscope (a–f). In addition, nuclear morphology was observed by fluorescence microscope after stained with Hoechst 33258 dyes (A–F). Scale bars: 20 μm in a–f; 10 μm in A–F.

mitochondrial depolarization could alter mitochondrial ROS generation. To investigate the effects of OGD-R and catalpol on astrocyte MMP, the fluorescent cationic dye, Rh 123, was used to determine changes in MMP. OGD-R induced a decrease in MMP, and this change was prevented by the addition of catalpol (Fig. 6).

4. Discussion

Studies on the pathomechanism of brain ischemia have mainly focused on neurons and thus, therapeutic strategies have been designed to counteract neuronal dysfunction. However, recent research indicates that astrocytes are critically affected in, and contribute to, the ischemic process (Gabryel and Trzeciak, 2001; Chen and Swanson, 2003). Thus, astrocytes may be targets and mediators of ischemia. In the present study, we used primary cultured astrocytes exposed to oxygen-glucose deprivation followed by reperfusion as an *in vitro* ischemic model to mimic ischemia-reperfusion injury and to assess the protective effects of catalpol on astrocytes against ischemia-induced injury. The experimental results demonstrated that

when astrocytes are exposed to OGD-R, a significant decrease in cell viability was seen, as previously observed by others (Gabryel et al., 2002; Han et al., 2000). Catalpol may significantly increase astrocyte survival. This finding indicates that catalpol is a potential protective agent against ischemic injury.

It is well known that ischemia, particularly post-ischemic reperfusion, enhances the formation of ROS in brain tissues (Oillet et al., 1996; Peters et al., 1998). Excessive production of ROS may induce cell damage either directly, through interacting and destroying cellular proteins, lipids and DNA, or indirectly, by affecting normal cellular signaling pathways and gene regulation (Kitagawa et al., 1990; Facchinetti et al., 1998; Lievre et al., 2000; Chan, 2001; Sugawara and Chan, 2003). A number of studies also have shown that antioxidants and ROS scavengers can reduce tissue damage following ischemic injury (Tagami et al., 1998; Cuzzocrea et al., 2000; Yamada et al., 2003). In this study, we have found that the exposure of astrocytes to OGD-R could result in significant increases in the levels of ROS and MDA, and this increase may be attenuated by catalpol. Cells also possess an array of

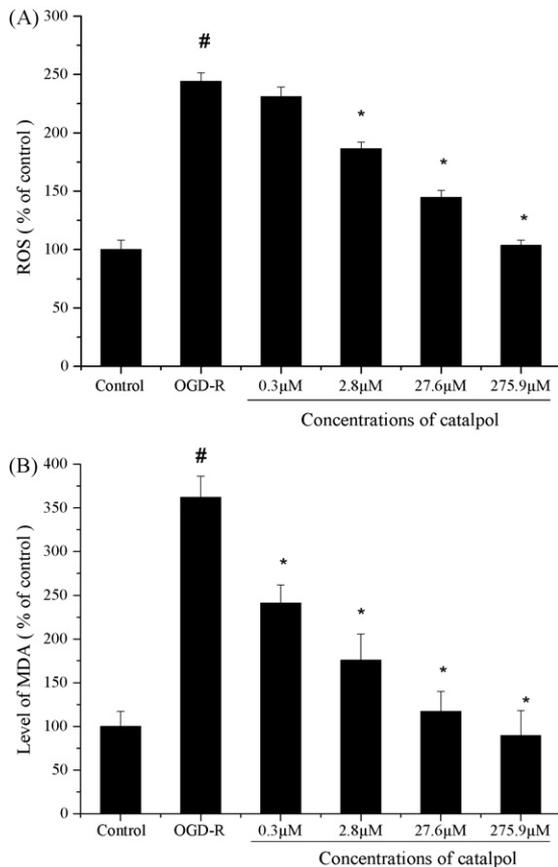


Fig. 3. Effects of catalpol on MDA level and ROS generation in cultured mouse astrocytes. Astrocytes exposed to normal oxygen and glucose or oxygen-glucose deprivation for 3 h followed by 20 h reoxygenation in the absence or presence of various concentrations (0.3 μM, 2.8 μM, 27.6 μM and 275.9 μM) of catalpol. The results are presented as percentage of control. Data are shown as mean ± S.D. from three separate experiments; the symbol (#) $P < 0.05$ as compared with control; the symbol (*) $P < 0.05$ as compared with OGD-R.

cellular defense systems, including antioxidant enzymes such as SOD, GPx, GSH and catalase (CAT), and low-molecular weight antioxidants such as GSH to scavenge and prevent damage caused by ROS. SOD is generally regarded as the primary line of defense against tissue and cellular damage caused by ROS. It catalyzes the dismutation of superoxide anion to peroxide. CAT and GPx provide a second of line of defense by dismutating peroxide into water and molecular oxygen. GPx converts any peroxide to water and molecular oxygen. However, GPx requires reduced-glutathione (GSH) to perform this function. GSH can scavenge ROS directly or indirectly, by the scavenging of peroxides by GPx. In addition, GSH can help recycle other antioxidants. There is considerable evidence of the involvement of these cellular defense systems in neuroprotection in ischemic insults (Yang et al., 1994; Chan et al., 1998; Wang et al., 2005). Astrocytes have higher antioxidant levels than neurons and can provide GSH precursors to neighboring neurons (Dringen et al., 2000). In vitro studies of a co-culture system of neurons and astrocytes show that astrocytes protect neurons from cell death induced by hydrogen peroxide (H_2O_2) and NO

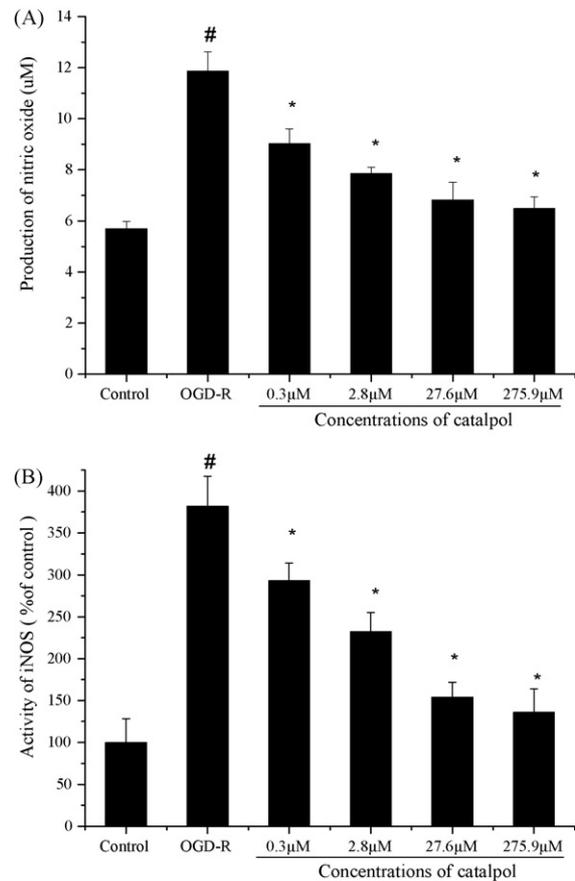


Fig. 4. Effects of catalpol on NO and iNOS in cultured mouse astrocytes. Astrocytes exposed to normal oxygen and glucose or oxygen-glucose deprivation for 3 h followed by 20 h reoxygenation in the absence or presence of various concentrations (0.3 μM, 2.8 μM, 27.6 μM and 275.9 μM) of catalpol. Data are shown as mean ± S.D. from three separate experiments; the symbol (#) $P < 0.05$ as compared with control; the symbol (*) $P < 0.05$ as compared with OGD-R.

(Desagher et al., 1996; Drukarch et al., 1998). These findings highlight the prominent role of astrocytes in the defense against oxidative stress-mediated neuronal cell death. However, astrocytes are critically affected in the ischemic process (Liu et al., 1999), which may have critical consequences on the progress and outcome of the ischemic lesion (Chen et al., 2001). In this study, we have examined the activities of SOD, GPx and the content of GSH to determine the effects of OGD-R on astrocytes and the protective effects of catalpol on astrocyte injury induced by OGD-R. Our results show that the activities of SOD, GPx and the level of GSH were decreased after OGD-R, which are consistent with others (Homi et al., 2002; Toyoda and Lee, 1997; Panickar and Norenberg, 2005). Catalpol seems to elevate astrocyte survival by decreasing the amount of ROS generated, stabilizing membranes and increasing the activities of SOD, GPx and the content of GSH.

Recent substantial evidence suggests that NO is involved in ischemic brain injury. Following brain ischemia, up-regulation of iNOS occurs, leading to an overproduction of NO that may damage the neural cells. It is likely that most of

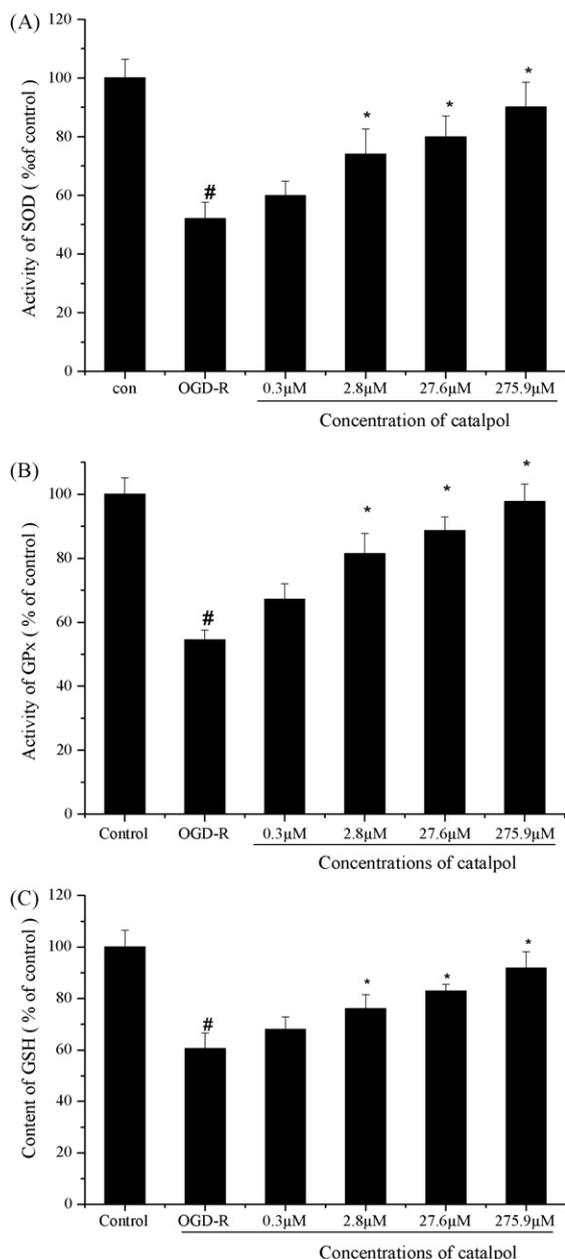


Fig. 5. Effects of catalpol on SOD and GSH-Px activities, GSH content in cultured mouse astrocytes. Astrocytes exposed to normal oxygen and glucose or oxygen-glucose deprivation for 3 h followed by 20 h reoxygenation in the absence or presence of various concentrations (0.3 μM, 2.8 μM, 27.6 μM and 275.9 μM) of catalpol. The results are presented as percentage of control. Data are shown as means ± S.D. from three separate experiments; the symbol (#) $P < 0.05$ as compared with control; the symbol (*) $P < 0.05$ as compared with OGD-R.

the neurotoxic actions of NO are mediated by peroxynitrite (ONOO⁻), the reaction product from NO and superoxide anion (Radi et al., 1991a,b; Cazeville et al., 1993; Dawson and Dawson, 1996). NO also inhibits the mitochondrial respiratory chain, which implicates the possible involvement of ATP loss and eventually leads to irreversible cellular damage (Radi et al., 1994). The NO generated by iNOS in astrocytes has an especially close relationship as a cytotoxic mediator under ischemic conditions (Iadecola et al., 1995a,b).

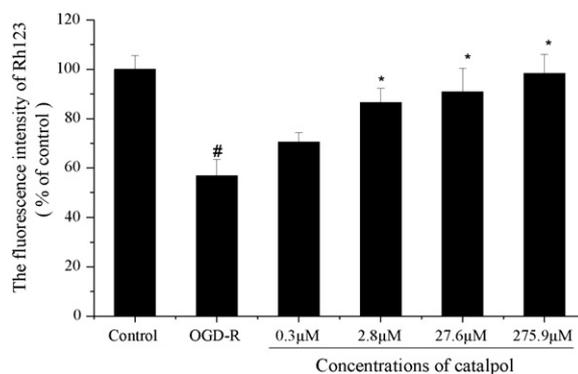


Fig. 6. Effect of catalpol on mitochondrial membrane potential in cultured mouse astrocytes. Astrocytes exposed to normal oxygen and glucose or oxygen-glucose deprivation for 3 h followed by 20 h reoxygenation in the absence or presence of various concentrations (0.3 μM, 2.8 μM, 27.6 μM and 275.9 μM) of catalpol. Mitochondrial membrane potentials are presented as percentage of the Rh 123 fluorescence intensity of control. Data are shown as mean ± S.D. from three separate experiments; the symbol (#) $P < 0.05$ as compared with control; the symbol (*) $P < 0.05$ as compared with OGD-R.

In iNOS knockout mice, infarct expansion after ischemia does not occur. Further, ischemia-induced brain damage was prevented by aminoguanidine, a selective iNOS inhibitor (Mori et al., 2001). These findings strongly suggest that glial-derived NO appear to contribute to delayed neurotoxicity following ischemic injury (Iadecola et al., 1995a,b; Radi et al., 1991a,b, 1994). In this study, we evaluated whether catalpol could prevent astrocyte damage by using an OGD-R model to study a NO-mediated mechanism. The experimental results showed that OGD-R-induced NO production and elevated the activity of iNOS in astrocytes. NO production induced by OGD-R and the increase of iNOS activity were markedly reduced by catalpol treatment. This data suggests that the protective effects of catalpol against OGD-R-induced damage in astrocytes may include attenuating NO production via down-regulation of iNOS activity.

The mitochondria are the primary intracellular source of ROS. Recent studies suggest that not only neurons but also astrocytes may experience mitochondrial dysfunction during ischemia-reperfusion (Bambrick et al., 2004; Dugan and Kim-Han, 2004). Mitochondrial dysfunction can contribute to cell death by reducing ATP production, increasing production of ROS, and releasing death regulatory and signaling molecules from the intermembrane space (Christophe and Nicolas, 2006). In this study, we also investigated the effects of OGD-R and catalpol on astrocytes MMP. OGD-R induced a decrease in MMP, as previously study by others, and this decrease was prevented by catalpol.

In conclusion, both biochemical and morphological studies show that catalpol protects against cell injury induced by OGD-R in a dose-response manner, in the range of 0.3–275.9 μM. Although other mechanisms cannot be excluded, it is possible that the protective effects in this study might be due in large part to the promotion of endogenous antioxidants and the reduction of ROS and nitric oxide formation by preservation of mitochondrial functions.

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