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Neuroprotective Effects of Berberine in Neurodegeneration Model Rats Induced by Ibotenic Acid

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Abstract: Berberine, an isoquinoline alkaloid found in *Coptidis Rhizoma* (goldenthread) extract, has multiple pharmacological effects such as anti-inflammatory, anti-microbial and anti-ischemic effects. In the present study, we examined the effects of berberine on neuronal survival and differentiation in a hippocampal precursor cell line and in the memory deficient rat model. Berberine increased in a dose dependent manner the survival of hippocampal precursor cells as well as differentiated cells. In addition, berberine promoted neuronal differentiation of hippocampal precursor cells. In the memory deficient rat model induced by stereotaxic injection of ibotenic acid into entorhinal cortex (Ibo model), hippocampal cells were increased about 2.7 fold in the pyramidal layer of CA1 region and about 2 fold in the dentate gyrus by administration of berberine after 2 weeks of ibotenic acid injection. Furthermore, neuronal cells immunoreactive to calbindin were increased in the hippocampus and entorhinal cortex area by administration of berberine. Taken together, these results suggest that berberine has neuroprotective effect in the Ibo model rat brain by promoting the neuronal survival and differentiation.

Key words: berberine, Alzheimer's disease, Ibo model, neural precursor cell, neuronal survival, neuronal differentiation, regeneration

INTRODUCTION

Alzheimer's disease (AD), which is characterized by senile plaques and neuronal dysfunction, is the most common dementia in the aging population. Neuronal cell death in the brain is believed to be a main cause of AD and is also found in most of degenerating brain diseases patients. Deficit of neuronal cells in entorhinal cortex and hippocampus region is an initial symptom of AD (Jensen et al., 1994; Selkoe, 1994; Ueki et al., 1997).

Berberine is an isoquinoline alkaloid compound, found in *Coptidis Rhizoma* (goldenthread) and *Hydrastis Canadensis* (goldenseal). It has multiple pharmacological and biological effects, such as antibiotic (Mirska et al., 1972), anti-inflammatory (Zhou and Mineshita, 2000; Marinova et al., 2000) and anti-hypolipidemic (Kong et al., 2004). Several groups also showed that berberine has a potential neuroprotective effect after transient forebrain ischemia (Wang et al., 2004; Yoo et al., 2006) and reduces beta amyloid (Aβ) levels by modulating APP processing (Zhu and Qian, 2006; Asai et al., 2007). Berberine has been also proposed to be beneficial to AD by improving expression of anti-inflammation factors and reducing Aβ levels by modulating APP processing (Zhu and Qian, 2006; Asai et al., 2007).

Here, we examined whether berberine has effects on neuronal survival and differentiation in hippocampal neural precursor cell, HiB5 and the differentiated HiB5 cells. We also examined its neuroprotective effect on the neuro-degeneration model rats induced by stereotaxic injection of ibotenic acid into entorhinal cortex (Ibo model), which suffer from neuronal loss via hippocampal perforant pathway defects similar to the brain of AD patients.

MATERIALS AND METHODS

Neuronal cell culture and cell viability assay

A rat neuronal stem cell line, HiB5 was maintained in DMEM containing 10% FBS and antibiotics at 33°C. For

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the experiments, cells (1×10^6) were changed to a chemically defined media N2 (except insulin) and incubated at 39°C. To induce cell differentiation, PDGF BB (20 ng/mL, Biosource, USA) was added one day after changing media to N2. Various concentrations of berberine chloride (0.05-6 μg/mL, Sigma, USA) were added to the medium and then the cells were cultured for 24 h after PDGF addition or 48 h after N2 change. Viability of the HiB5 cells was measured by using a MTT assay. HiB5 cells (8×10^3) in a 96-well plate were grown for 48 h at 33°C, and moved to N2 medium and incubated at 39°C. MTT assay was carried out as described in a previous report (Joung et al., 2005).

Immunocytochemistry

To evaluate differentiation effect of berberine with or without PDGF BB, HiB5 cells were cultured for 48 h after PDGF addition or 72 h after N2 change without PDGF. The cells grown on coverslips in a 24 well plate (4×10^4 cells) were fixed in 4% paraformaldehyde (PFA) and permeabilized in 0.5% Triton X-100 for 10 min. Cells were then incubated at room temperature in PBS containing blocking serum (5% normal goat serum) and washed with PBS. Cells were then incubated for overnight at 4°C with primary antibody against Neurofilament (1:1000; Covance, USA). Next day cells were washed and treated with FITC-conjugated secondary antibody (1:500; Jackson Lab, USA) for 1 h at the room temperature and followed by the incubation with propodium iodide (1 μg/mL) for 5 min at 4°C. Images were analyzed using LSM 510 confocal microscope (Carl Zeiss, Germany).

Animals

Male Sprague-Dawley rats (Adult 250-270 g) were purchased from the Orient Co., Ltd, a branch of Charles River Laboratories (Seoul, Korea). Animals were housed 2 or 3 per cage, allowed access to water and food *ad libitum*, and maintained under a constant temperature (23±1°C), humidity (60±10%) and a 12 h light/dark cycle (light on 07.00-19.00 h). Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyunghee University, Korea.

Surgeries on the memory deficient rat model

The surgery protocol was followed as previously described with modifications (Ueki et al., 1994; Park et al., 2000). Briefly, the rats were anesthetized with equithensin (0.35 M sodium pentobarbital, 250 mM chloral hydrate, 85 mM $MgSO_4$, 40% prophylene glycol in 10% ethanol, 2 mL/kg). Ibotenic acid (1.5 μL per animal, 1 mg/mL, Sigma) was injected into the entorhinal cortex as following: rats were placed in a Stereotaxic device (Stoelting Co., Wood Dale, IL) with the incisor bar 3.4 mm below the interaural line and the needle was positioned at an angle of 10° right to the midsaggital plane. Ibotenic acid was injected 3 times, 0.35 μL/min speed (first, AP: −8.4 mm, ML: −4.8 mm, DV: −4.6--4.8 mm; second, AP: −8.4 mm, ML: −4.8 mm, DV: −2.6--2.8 mm; third, AP: −8.8 mm, ML: −3.65 mm, DV: −4.8 mm). As a sham group, saline was injected instead of ibotenic acid and as a control group, ibotenic acid was injected. Berberine chloride stock solution for animal experiments was prepared by dissolving in saline to make 100 mg/mL.

One week after ibotenic acid injection, the rats were intraperitoneally injected with berberine (50 mg/kg) or vehicle once a day for a week. Two week after the initiation of the drug administration, the brains of the rats were immunostained.

Animal groups were 3 groups: 1) Ibo model rats administrated with vehicle (saline) once a day for a week, 2) Ibo model rats administrated with berberine once a day for a week and 3) saline injected sham group was administrated with berberine once a day for a week.

Tissue Preparation

Following perfusion with 4% PFA in PBS, the brains were removed and fixed with 4% PFA in PBS for 4 h. The brains were cryoprotected in 30% sucrose-PBS and then frozen with OCT compound and stored at 80°C until processed. Brain tissue blocks were cryosectioned through the coronal plane at a thickness of 35 μm. Sections were stored at 4°C in storing solution (30% glycerol, 30% ethylene glycol in PBS). Before staining, the sections were thoroughly rinsed in PBS.

Immunohistochemistry

Immunostaining analysis of brain slices was carried out as previously described with modifications (Kwon, 1997). The cryosectioned brain slices (35 μm) were permeabilized in 0.5% Triton-X100 for 20 min and blocked in 15% normal serum with 3% BSA and 0.1% Triton X-100 for 2 h in free floating condition. The sections were incubated for 16 h at 4°C with antibodies against calbindin D28k (1:1000; Chemicon, USA). Sections were washed with PBS and incubated with FITC-conjugated anti-mouse donkey antibody (1:500; Jackson Lab) for 50 min at room temperature. Sections were washed with PBS and then counterstained with Mayer's hematoxylin for 1 min. Sections were mounted with Dako mounting solution and examined under a confocal laser microscope (LSM10, Carl Zeiss).

Quantification of Data and Statistical Analysis

Hematoxylin stained cells were counted in the CA1 field, dentate gyrus (AP: Bregma −4.5--4.3 mm) and entorhinal

cortex region (AP: Bregma −8.3--8.5 mm) of every fifth cryo-section. Images of hippocampal coronal-sections for staining analysis were taken for assessing the number of the stained cells in the CA1, dentate gyrus and entorhinal regions of one brain slice under 100× magnifications. Three animals per group were processed, and values were expressed as means±S.E.M.

The number of DAPI stained cells and cells immuno-reactive to pan-neurofilament in the cultured HiB5 cells were counted and the averages of cell numbers in a microscopic field (the area=211.6 mm^2) are represented as % of control cells.

The statistical analyses of the data were performed by one-way analysis of variance and $P<0.05$ was taken as being significant.

RESULTS

Effects of berberine on the survival of neural precursor cells and differentiated neuronal cells

HiB5 cells are a neural precursor cell line isolated from embryonic day-16 rat hippocampus. Since they were immortalized by the temperature sensitive SV40 large T antigen (Renfranz et al., 1991), they grow at 33°C and express nestin, a stem cell marker. In serum-free N2 medium at a nonpermissive temperature of 39°C, however, the HiB5 cells stop growing and only 30~40% of them are able to survive and extend neurite-like structure. Previously we showed that PDGF induces the differentiation of HiB5 precursor cells to neuronal marker expressing cells (Joung et al., 2005; Lim et al., 2007). PDGF promotes the survival of the differentiated HiB5 cells as well.

In order to investigate the effect of berberine on survival of neural precursor cells and differentiated neuronal cells, we measured cell survival of HiB5 precursor cells before and after the differentiation by MTT assay (Fig. 1A and B). The result shows that cell survival of HiB5 precursor cells was enhanced by berberine in a dose-dependent manner (Fig. 1A). The maximal effect of berberine was found at 3 μg/mL. Berberine markedly enhanced the survival of the differentiated HiB5 cells after the addition of PDGF (Fig. 1B). Berberine at a concentration of 3 μg/mL increased the cell survival more than 2.5 fold and this value is higher than that promoted by PDGF. These results suggest that berberine promotes the survival of both neural precursor cells and differentiated neuronal cells and has an additive effect with PDGF.

Berberine promotes neuronal differentiation of hippocampal precursor cells

We next asked whether berberine effectively enhances differentiation of the surviving HiB5 cells because more cells initiated elongation of their cell bodies under the

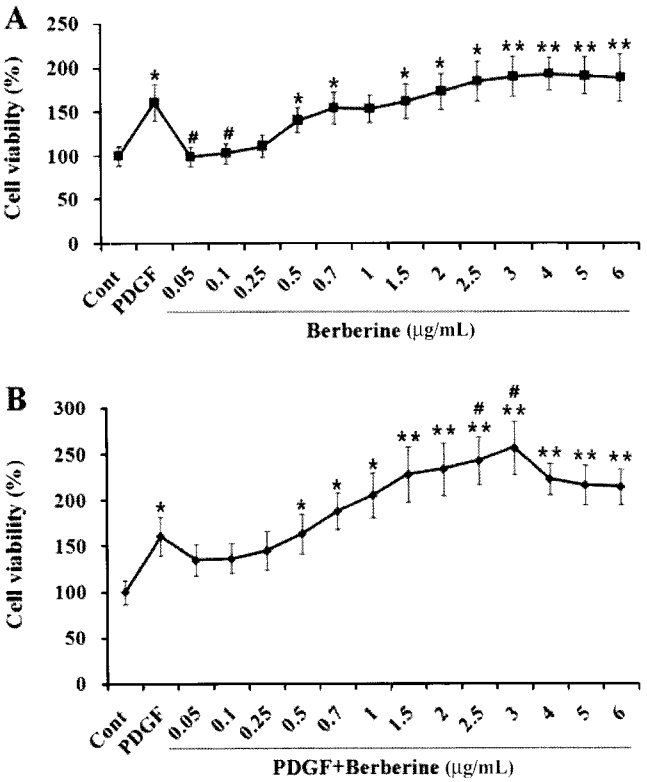

Fig. 1. Effect of berberine on the cell survival of neural precursor and the differentiated HiB5 cells. Berberine (0.05-6 μg/mL) was added to a hippocampal precursor (A) and the differentiated neuronal cells, HiB5(B). Differentiation of HiB5 cells was induced with PDGF (20 ng/mL) for 2 days (B). Cell survival was measured by MTT assay. Cell viability was normalized to the control. Data from five independent experiments were subjected to statistical analysis. Shown as means ±S.E.M. *P<0.05 and **P<0.01 compared with the control. #P<0.05 compared with the PDGF alone.

addition of berberine. Based on the results from MTT assay, we chose a concentration of berberine, 3 μg/mL as the optimal concentration for further investigation. Under the differentiation condition, untreated HiB5 cells showed a poor rate of differentiation. On the other hand, most of the surviving HiB5 cells treated with PDGF showed the changes of their morphologies characteristic to neuronal cells such as the neurite outgrowth and increased expression of neurofilament (Fig. 2A).

We treated HiB5 cells in the differentiation condition with berberine in the presence or absence of PDGF, and immunostained the cells with anti-neurofilament antibody. With addition of berberine in the absence of PDGF, the percentages of neurofilament expressing cells were increased about 30% which is more than 2 fold compared to the control (Fig. 2B), suggesting that berberine promotes neuronal differentiation of hippocampal precursor cells. When differentiation of HiB5 cells was previously induced by PDGF, however, berberine did not increase their differentiation rate more than that of PDGF-alone treated cells.

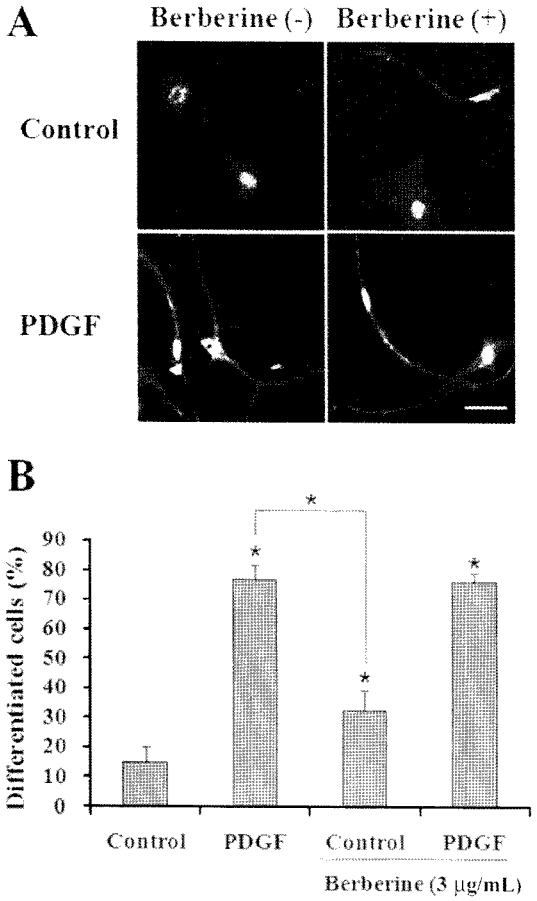

Fig. 2. Promotion of the differentiation of HiB5 cells by addition of berberine. (A) The differentiation of HiB5 cells into neuronal cell was assessed by immunocytochemical staining with anti-neurofilament antibody (green). Differentiation of HiB5 cells was induced in the presence of berberine (3 μg/mL) and PDGF (20ng/mL) for 2 days. Nucleus was counterstained with propidium iodide (red). Scale bar =50 μm. (B) Quantification of neurofilament immunoreactive cells. Data from five independent experiments were subjected to statistical analysis. Shown as means±S.E.M. *P>0.05 compared with the control.

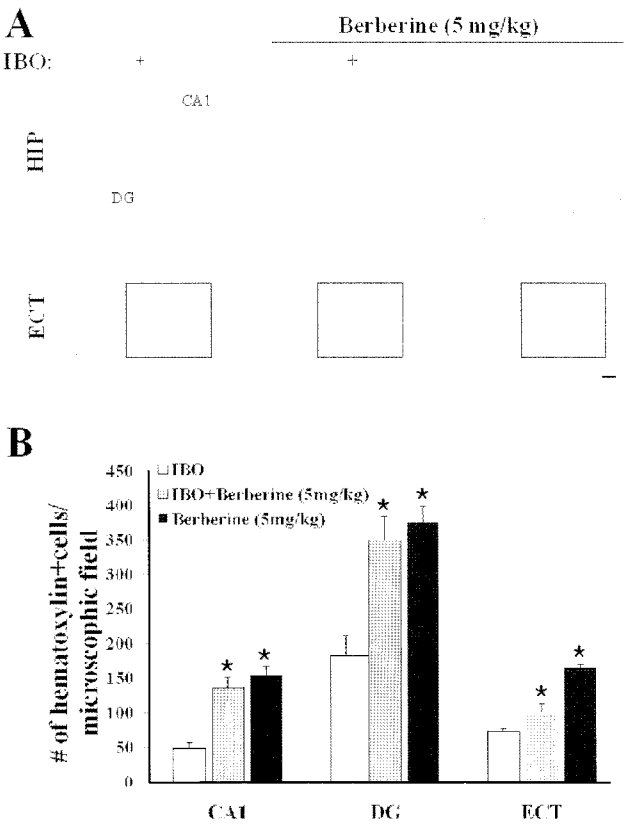

Fig. 3. Number of hippocampal cells increased in the Ibo model rats by administration of berberine. Ibo model rats were generated by stereotaxic injection of ibotenic acid. After intraperitoneal administration of berebrine (5 mg/kg) for 1 week which is two weeks after ibotenic acid injection, brain sections were stained with hematoxylin. (A) Representative images of brain sections on the hippocampal area (HIP) and the entorhinal cortex area (ECT). CA1 and dentate gyrus (DG) in the hippocampal area were indicated. Scale bar=200 μm (x100). (B) Numbers of hematoxylin reactive cells in CA1, dentate gyrus (DG) in the hippocampal area and the entorhinal cortex (ECT) were counted (square box images). Data from three animals were subjected to statistical analysis. Shown as means±S.E.M. *P<0.05 compared with the Ibo model.

Effect of berberine on the hippocampal cell survival of the Ibo model rats

Having established that berberine promotes cell survival and differentiation of HiB5 cells, we next investigated whether berberine has neuroprotective effect on the Ibo model rats. In order to generate the Ibo model rats, adult rats were injected stereotaxically with ibotenic acid into the entorhinal cortex. This resulted in the neuronal loss in the entorhinal cortex (ECT) and then in the CA1 regions and dentate gyrus of the hippocampus where cellular damage is first found in brains of AD patients (Ueki et al., 1994; Park et al., 2000).

To elucidate neuroprotective effect of berberine on the Ibo model rats, we examined the numbers of hippocampal cells in the CA1 pyramidal layer, dentate gyrus (DG), and entorhinal cortex regions using hematoxylin staining. After intraperitoneal administration of berebrine for one week which is two weeks after ibotenic acid injection, animals were perfused and their brains were fixed. Brain tissues were cryosectioned through the coronal plane and then stained with Mayer's hematoxylin. The number of hematoxylin stained cells in the damaged region was significantly decreased in the group treated with ibotenic acid. The reduction in cell numbers brought by ibotenic acid insult was significantly improved by the administration of berberine (Fig. 3). Administration of berberine increased the number of hemotoxylin stained cells in the CA1 area by 2.7-fold, in DG area by 1.9-fold, and in ECT area by 1.3-fold compared to the Ibo control group (Fig. 3B). Berberine administration also promoted cell survival in the ECT slices of sham normal rats which were injected with saline instead of ibotenic acid (data not shown).

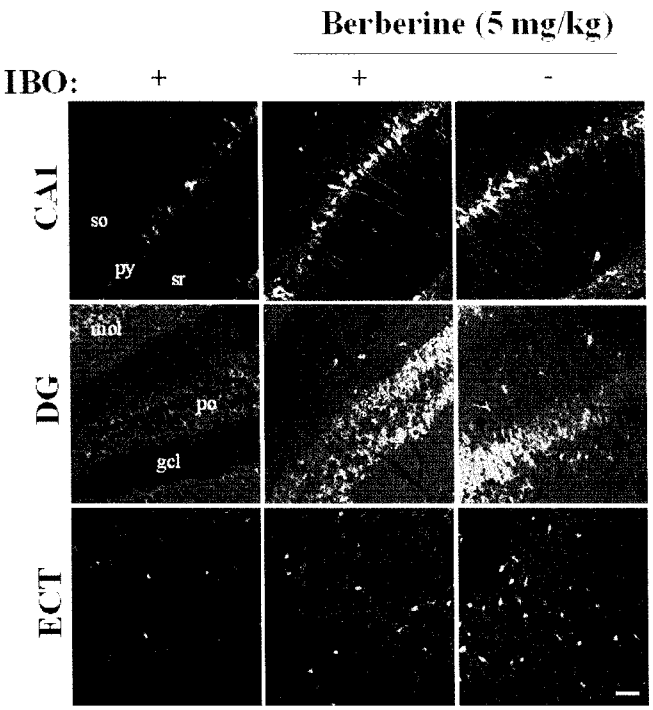

Fig. 4. Calbindin immunoreactive cells increased by administration of berberine in the hippocampal and the entorhinal cortex area in the Ibo model rats. Ibo model rats were treated with berberine (5 mg/kg) intraperitoneally for 1 week. Brain sections were immunostained with anti-calbindin antibodies. Representative images of brain sections on CA1 and dentate gyrus (DG) in the hippocampal area and the entorhinal cortex (ECT) were presented. So, stratum oriens; py, pyramidal cell layer of CA1; sr, stratum radiatum; mol, molecular layer of dentate gyrus; gcl, granule cell layer of dentate gyrus; po, polymorph layer. Scale bar=100 μm (x100).

Protection of calbindin-positive neurons by berberine in the Ibo model rats

A neuronal calbindin D28k is a marker protein for neuronal cells and often used as a marker for cholinergic neurons (Geula et al., 2003; Dénes and Gábriel, 2004). In order to examine whether berberine promotes survival of neuronal cells in the Ibo model rats, we immunostained the rat brain slices including hippocampal CA1, dentate gyrus and entorhinal cortex with anti-neuronal calbindin D28k antibodies and scanned them under a confocal laser scanning microscope.

As expectedly, the number of calbindin-positive cells in the rat brain slices including hippocampal CA1, dentate gyrus and ECT was decreased strikingly in each region by ibotenic acid injection. When berberine was administrated to ibotenic acid injected rats, the number of calbindin immunostained cells increased about 4.4-fold in the CA1 area, about 5-fold in DG area, and about 3.3-fold in ECT area compared to the Ibo control group, indicating it recovered almost to the level of the berberine-only treated group (Fig. 4). The calbindin-positive cells found in the berberine treated group exhibited longer and more neurites compared to the ibotenic acid injected group. This observation clearly indicates that berberine improves the survival of neuronal cells in the hippocampus and entorhinal cortex of Ibo model rats.

DISCUSSION

Previous studies have shown that berberine has multiple pharmacological actions including anti-inflammatory and anti-amnesic effect but the underlying mechanisms are not clear (Mirska et al., 1972; Zhou and Mineshita, 2000; Yoo et al., 2006). In this study, we showed that berberine promotes cell survival and differentiation of hippocampal precursor cells. In addition, intraperitoneal administration of berberine increased the numbers of neuronal cells expressing calbindin in the Ibo model rats. Since calbindin is a marker protein for neuronal cells, this result clearly indicates that berberine is able to improve the survival of neuronal cells in the hippocampus and entorhinal cortex of Ibo model rats. The Ibo model rats show neuronal loss and memory deficiency because cell death caused by ibotenic acid in the entorhinal cortex reduces survival of hippocampal pyramidal cells and granule cells in dentate gyrus retrogradely via hippocampal perforant pathway. These symptoms are similar to the brain of early AD patients. AD patients also show defects of cholinergic system in the cortical and the hippocampal areas (Herholz et al., 2008). The increase in neuronal cell numbers by administration of berberine in the Ibo model rats could be caused by that berberine improves neuronal regeneration through the increase of cell survival and differentiation of endogenous neural stem cells and the differentiated cells. Another possibility is that berberine blocks the retrograde cell death in the rat hippocampus which is induced by ibotenic acid injection into ECT. In the hippocampal precursor cell line, HiB5 we found that berberine facilitated the survival of neural precursor cells and the differentiated neuronal cells immunoreactive to neurofilament. Berberine also promoted the differentiation of neural precursor cells, but it did not promote the differentiated neuronal cells additionally. These data support the idea that the increase in number of neuronal cells in the hippocampus and ECT is due to the new born cells that their survival and differentiation is improved by berberine. It is interesting that effect of berberine was additive on the cell survival of differentiated HiB5 cells but not on the differentiation when it was already induced by PDGF. This suggests berberine promoted the differentiation of HiB5 precursor cells via the same pathway as PDGF did. There is a report that addition of berberine enhanced the expression of signal transducing proteins, Gαq/11 and of phospholipase-C β1/4 in the differentiated human CNS precursor, NT2 cells (Novak et al., 2000), a mechanism controlling HiB5 cell differentaiation by addition of berberine remains to be

elucidated.

Recently, it has been demonstrated that berberine reduce reactive oxygen species (ROS) generation and subsequent release of cytochrome c and apoptosis-inducing factors (AIFs) in PC12 cells insulted by oxygen-glucose deprivation (Zhou et al., 2008). Neuroprotective effects of berberine against ischemic damage are proposed to be via the reduction of N-methyl-D-aspartate (NMDA)-receptor 1 activity (Yoo et al., 2006). Considering that an excessive NMDA-receptor activation during the cerebral ischemia could induce mitochondrial dysfunction and ROS production and that the insult of ibotenic acid in the Ibo model rats is primarily mediated by hyper-activation of glutamate receptors, the neuroprotective effect of berberine against neuronal cell death in the hippocampus caused by ibotenic acid could be mediated by blocking ROS production via regulating glutamate receptor. In addition, anti-inflammation effect of berberine probably contributes to improve the neuronal cell survival. Berberine also blocks potassium channels in acutely isolated CA1 pyramidal neurons of rat hippocampus (Wang et al., 2004). Considering the recent demonstration that mutations in voltage-gated potassium channel KCNC3 could cause adult-onset ataxia (Waters et al., 2006), we cannot exclude the possibility that berberine modulates potassium channels.

Recently, natural herb extracts have been investigated for use as therapeutic reagents to treat neurodegenerative disorders. Several groups have already identified candidate herbs that may prove to be useful (Asai et al., 2007, Dhanasekaran et al., 2007, Kim et al., 2007). Berberine is a major alkaloidal component of *Coptidis Rhizoma* (goldenthread), a herb widely used in oriental medicine. Our results suggest that berberine has significant neuroprotective effects and it has a vast potential for the therapeutic utility for neurodegenerative diseases such as neuropsychiatric disorder and dementia.

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