Effects of Scutellarin on Apoptosis Induced by Cobalt Chloride in PC12 Cells

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Abstract

The present study investigated the protective effects of scutellarin on cobalt chloride ($CoCl_2$)-induced apoptosis in PC12 cells. Incubation of PC12 cells with 500 μM $CoCl_2$ for 24 h resulted in significant apoptosis as evaluated by the crystal violet, electron microscopy and flow cytometry assays. The increase of caspase-3 activity, decrease of Bcl-X_L expression, phosphorylation of p38 mitogen-activated protein kinase (MAPK) and accumulation of intracellular reactive oxygen species (ROS) were also seen in $CoCl_2$-treated PC12 cells. Scutellarin at 0.1, 1 and 10 μM significantly protected against the apoptotic cell death induced by $CoCl_2$. Scutellarin decreased caspase-3 activity, increased Bcl-X_L expression, inhibited p38 phosphorylation and attenuated ROS production. These results demonstrate that scutellarin can protect PC12 cells from cobalt chloride induced apoptosis by scavenging ROS, inhibiting p38 phosphorylation, up-regulating Bcl-X_L expression and decreasing caspase-3 activity, and may reduce the cellular damage in pathological conditions associated with hypoxia-mediated neuronal injury.

Key Words: scutellarin, flavone glycoside, PC12 cells, cobalt chloride, apoptosis, reactive oxygen species, caspase-3, p38 MAPK

Introduction

Scutellarin (Fig. 1), a novel flavonoid, is the major active ingredient in breviscapine. Breviscapine is a mixture extracted from Erigeron breviscapus (Vant.) Hand.-Mazz, a plant used in Chinese herbal medicine. In China, breviscapine injections are widely used for the treatment of ischemic cerebrovascular diseases. In recent years, studies have shown that scutellarin can attenuate neuronal damage induced by hydrogen peroxide (19), oxidative glutamate (13) and cerebral ischemia/reperfusion (15). However, whether scutellarin is capable of protecting neuron from apoptosis, especially from hypoxia-induced apoptosis, remains unclear.

Apoptosis is shown to play a crucial role in many physiological and pathological processes (1). Evidence is accumulating that apoptosis may be implicated in neural disorders including hypoxia. Hypoxia-induced cell death is a major concern in various clinical entities, such as ischemic disease, organ transplantation and other disease (26). There are many reports showing that cobalt chloride ($CoCl_2$) could mimic the hypoxic responses in cultured cells (5, 6, 9, 10). Studies have also shown that $CoCl_2$ can induce apoptosis in PC12 cell, a commonly used cell line for investigating neuronal cell death (23), and that the $CoCl_2$-treated PC12 cells may serve as a simple *in vitro* model for the study of the mechanism of hypoxia-linked neural disorders (32, 33). In this study, $CoCl_2$-treated PC12 cells were chosen to

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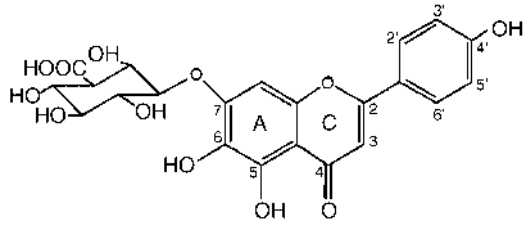

Fig. 1. Chemical structure of scutellarin.

investigate the effect of scutellarin on neuronal apoptosis in primary culture.

Materials and Methods

Drugs and Reagents

Scutellarin (purity 96.6%, HPLC) was provided by Changxing Pharmaceutical Co. Ltd. (Jinan, China). Cobalt chloride and poly-D-lysine (MW 70,000-150,000) were purchased from Sigma Chemical Co. (St, Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Gibco BRL (Life Technologies, Paisley, Scotland, UK). Rabbit anti-Bax, rabbit anti-Bcl-X_L, goat anti-Bcl-2, rabbit anti-p38 and mouse anti-P-p38 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular Probes (Eugene, OR, USA). Colorimetric CaspACE™ assay system was obtained from Promega (Madison, WI, USA). All other reagents and solvents used in experiments were of analytical grade.

Cell Culture

The clonal rat pheochromocytoma cell line, PC12, was obtained from Shanghai Institute of Cell Biology (Shanghai, China) and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM supplemented with 10%(V/V) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. For all experiments, PC12 cells were plated on poly-D-lysine-coated plastic tissue culture dishes for 18-24 h before treatments.

Cell Viability

PC12 cells were seeded at 50,000 cells/ml in 96-well plates for the determination of cell viability. After removing of cell culture medium, the cells were exposed to 2% crystal violet in 20% methanol for 15 min. Thereafter, cells were washed with distilled water, air dried for 24 h, and lysed with 0.1 M citrate buffer. The optical density was measured photometrically on a Dyratech plate photometer. Background readings were performed by staining of poly-D-lysine-precoated cell-free wells.

Electron Microscopy

Cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4, postfixed in 1% osmium tetroxide, followed by en bloc staining with 2% uranyl acetate. Samples were dehydrated serially through increasing concentrations of ethanol and embedded in Epon. Sections were examined with an electron microscope.

Flow Cytometric Analysis

Cells were collected and washed with PBS, then fixed in 70% ethanol for 2 h at 4°C. Thereafter, cells were washed with PBS and re-suspended in 0.2 ml PBS containing 1 mg/ml propidium iodide (PI) and 0.1% Triton X-100. Samples were kept in the dark for 30 min at 4°C and subsequently for flow cytometric analysis of percentage of apoptotic cells in total.

Western Blot Analysis

Cells were collected and washed with PBS, then lysed in 120 µl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1.55% DTT) and boiled for 10 minutes. Total cell lysates (30 µg of protein) were separated by SDS-PAGE (12% for Bax, Bcl-2, Bcl-X_L; 8% for p38, P-p38) and transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, USA). Immunodetection was performed with appropriate antibody using an enhanced chemiluminescence (ECL) system (Amersham).

Caspase-3 Assay

Caspase-3 activity was determined by a caspase-3 assay kit. In brief, cells were collected and washed once with ice-cold PBS and lysed with cell lysis buffer containing 1% Triton X-100, 50 mM-HCl (pH 7.4), 1 mM EDTA, and 1 mM PMSF. Complete lysis was performed by three rounds of freeze-thawing. Lysates were incubated on ice for 15 min and centrifuged at 15,000 × g for 20 min at 4°C. The supernatant fractions were collected and incubated with the caspase-3 substrate, acetyl- Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD pNA) at 37°C for 4 h. caspase-3 activity was assessed by measuring the absorbance at a wavelength of 405 nm with a spectrophotometer.

Measurement of ROS Generation

We determined intracellular ROS levels by

staining cells with 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), which was oxidized to highly fluorescent dichlorofluorescein (DCF) by ROS. Cells were harvested and preincubated with 10 μM H_2DCFDA for 30 min in PBS containing 5 mM glucose at 37°C. After twice washing, cells were re-suspended with PBS containing 5 mM glucose. Measurement of fluorescence intensity is carried out in a Hitachi F4010 spectrofluorometer, with an excitation wavelength at 488 nm and with emission at 525 nm. Temperature was maintained at 37°C throughout the experiment.

Statistical Analysis

Data were expressed as the mean ± SD. Statistical significance analysis was determined using Student's *t*-test, and a *P* value of < 0.05 was considered statistically significant.

Results

Scutellarin Protects PC12 Cells from Apoptosis Induced by $CoCl_2$

Compared with control group, PC12 cells viability decreased after treatment with 500 μM $CoCl_2$ for 24 h (Fig. 2). Preincubating with indicated amounts of scutellarin for 2 hours resulted in $CoCl_2$-treated PC12 cells viability increasing in a dose-dependent manner (Fig. 2).

To confirm the cell viability assessed with the crystal violet assay, we also used flow cytometric analysis. Treatment with 500 μM $CoCl_2$ for 24 h caused an obvious increasing percentage of apoptosis population in PC12 cells. (Fig. 3, A and B). Preincubating with indicated amounts of scutellarin for 2 h resulted in the percentage of apoptosis population decreasing in a dose-dependent manner (Fig. 3, C, D, and E).

The highly condensed and fragmented nuclei are the typical characteristics of apoptosis. After treatment with 500 μM $CoCl_2$ for 24 h, electron microscopy showed the blebbing of the cell surface and condensed chromatin localized to the inner side of an intact nuclear membrane (Fig. 4B). Preincubating with indicated amounts of scutellarin for 2 h improved the morphological changes significantly (Fig. 4, C, D, and E).

Scutellarin Inhibits the Activity of Caspase-3 in $CoCl_2$-Induced Apoptosis in PC12 Cells

Caspase-3 is a key protease during apoptosis induced by various stimuli. The fluorescent peptide substrate Ac-DEVD pNA was used to examine the activity of caspase-3 in the cell lysates. After treatment with 500 μM $CoCl_2$ for 8 h, the caspase-3 activity that resulted in the cleavage of the peptide substrate

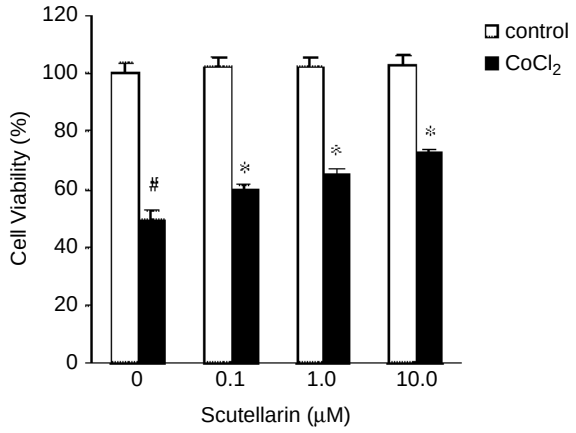

Fig. 2. Effects of scutellarin on cell viability after treatment with 500 μM $CoCl_2$ for 24 h in PC12 cells (mean ± SD, n = 6). $^{\#}P < 0.01$, significant difference between PC12 cells in the absence or presence of $CoCl_2$ by *t*-test. $*P < 0.01$, significant difference between $CoCl_2$-treated PC12 cells in the absence or presence of scutellarin by *t*-test.

Ac-DEVD *p*NA increased significantly (Fig. 5). Preincubating with indicated amounts of scutellarin for 2 h decreased the activity of caspase-3 in a dose-dependent manner (Fig. 5).

Scutellarin Up-Regulates Bcl-X_L in $CoCl_2$-Induced Apoptosis in PC12 cells

Using Western blot, we examined the expression of Bax, Bcl-2 and Bcl-X_L, as prototypes of the regulatory proteins of apoptosis. After treatment with 500 μM $CoCl_2$ for 24 h, the expression of Bcl-X_L decreased obviously (Fig. 6). Preincubating with indicated amounts of scutellarin for 2 h increased the expression of Bcl-X_L in a dose-dependent manner (Fig. 6). We failed to observe any change of Bax (Fig. 6). Regardless of $CoCl_2$ treatment or not, Bcl-2 was not expressed in PC12 cells (data not shown).

Scutellarin Inhibits the Phosphorylation of p38 MAPK in $CoCl_2$-Induced Apoptosis in PC12 Cells

Our previous studies have demonstrated that p38 is phosphorylated during apoptotic cell death induced by $CoCl_2$ (33). Using Western blot, we examined the effect of scutellarin on the phosphorylation of p38. Results showed that treatment with 500 μM $CoCl_2$ for 12 h caused a significant phosphorylation of p38 (Fig. 7) and preincubating with indicated amounts of scutellarin for 2 h inhibited the phosphorylation in a dose-dependent manner (Fig.7).

Scutellarin Decreases the ROS Production in $CoCl_2$-Induced Apoptosis in PC12 cells

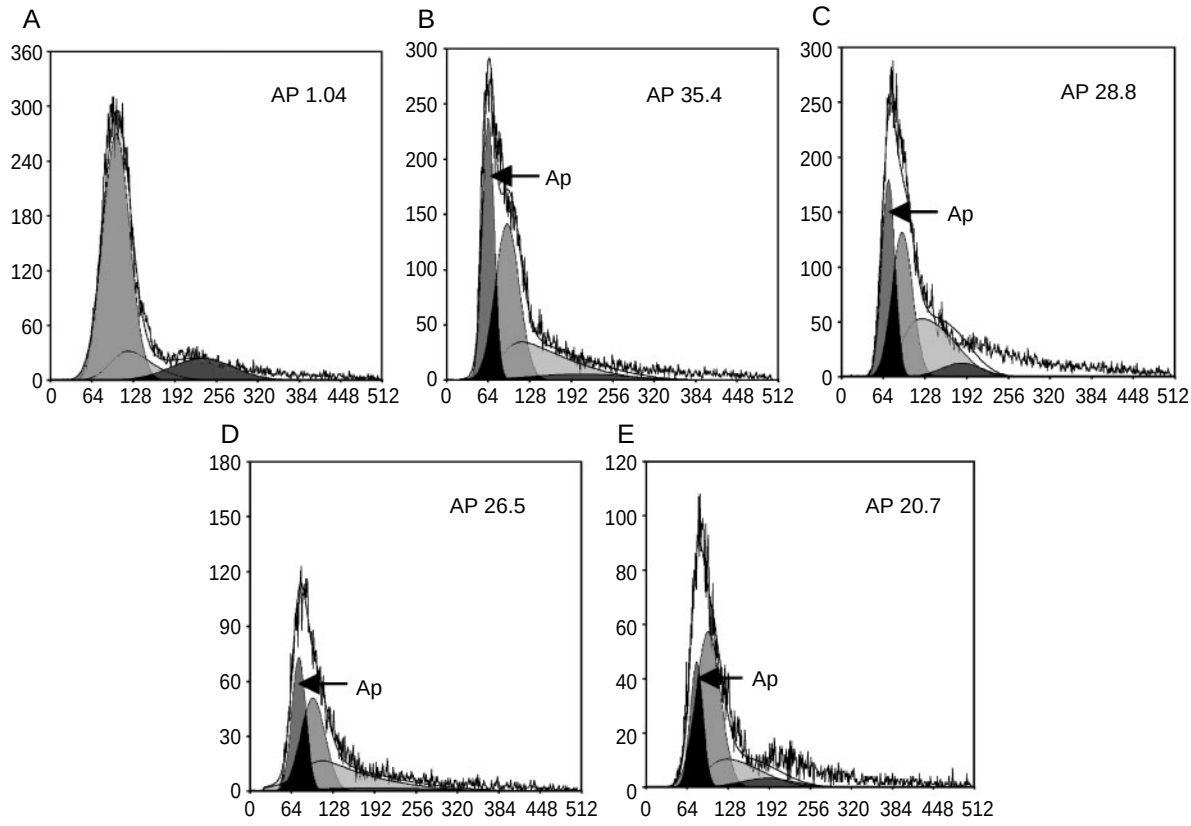

Fig. 3. Effects of scutellarin on apoptotic cell populations in PC12 cells after treatment with 500 μM $CoCl_2$ for 24 h using flow cytometric analysis. Percentage of apoptosis (AP) population is shown. The results are obtained from a representative experiment among three independent experiments. The apoptotic cell populations of the untreated cells (A) are only 1.04%, whereas the $CoCl_2$-treated cells (B) show marked growth of the apoptotic cells. Scutellarin at 0.1, 1.0 and 10.0 μM decrease the apoptosis significantly (C, D, and E).

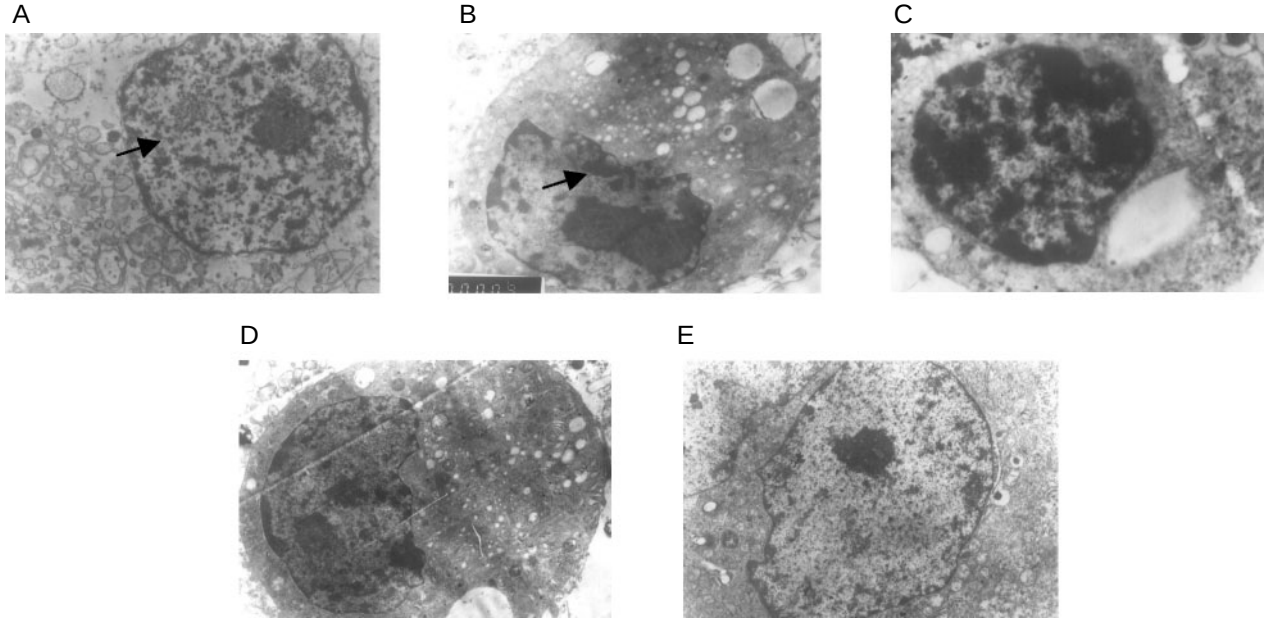

Fig. 4. Effects of scutellarin on morphological changes of PC12 cells after treatment with 500 μM $CoCl_2$ for 24 h using with electron microscopy. The untreated cells (A) have large nuclei and the heterochromatin is scanty, whereas the $CoCl_2$–treated cells (B) show marked chromatin condensation within an intact nuclear envelop and bleb of cell surface. Scutellarin at 0.1, 1 and 10 μM improved the morphological changes significantly (C, D, and E).

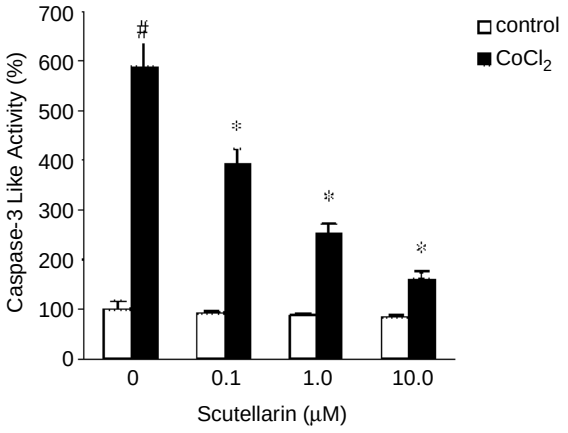

Fig. 5. Effects of scutellarin on caspase-3 activity after treatment with 500 μM $CoCl_2$ for 8 h in PC12 cells (mean ± SD, n = 6). $^{\#}P < 0.01$, significant difference between PC12 cells in the absence or presence of $CoCl_2$ by *t*-test. $^{*}P < 0.01$, significant difference between $CoCl_2$-treated PC12 cells in the absence or presence of scutellarin by *t*-test.

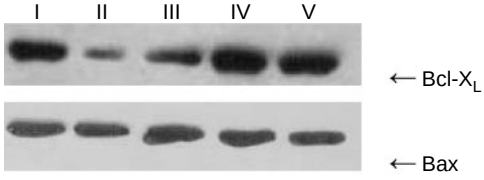

Fig. 6. Effects of scutellarin on Bcl-X_L and Bax after treatment with 500 μM $CoCl_2$ for 24 h in PC12 cells, n = 3. The protein level of Bcl-X_L and Bax were determined by Western blot. Lane I: control; lane II: $CoCl_2$; lane III, IV, V: scutellarin 0.1, 1, 10 μM.

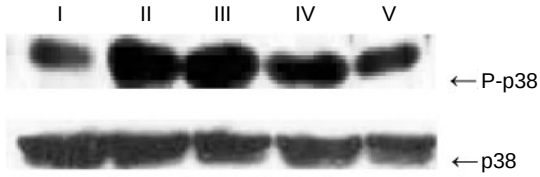

Fig. 7. Effects of scutellarin on the activity of p38 MAPK after treatment with 500 μM $CoCl_2$ for 12 h in PC12 cells, n = 3. The protein levels of P-p38 as well as p38 were determined by Western blot. Lane I: control; lane II: $CoCl_2$; lane III, IV, V: scutellarin 0.1, 1, 10 μM.

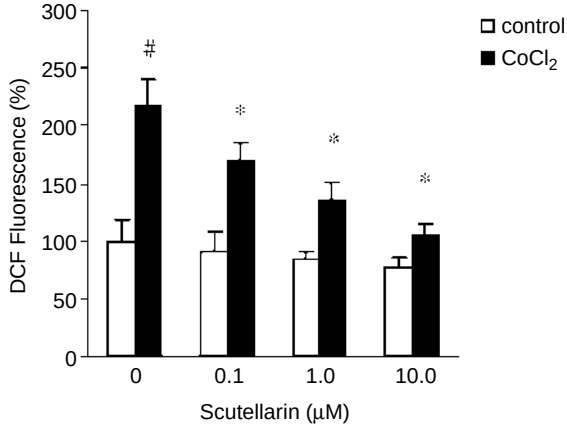

Fig. 8. Effects of scutellarin on ROS production after treatment with 500 μM $CoCl_2$ for 1 h in PC12 cells (mean ± SD, n = 6). $^{\#}P < 0.01$, significant difference between PC12 cells in the absence or presence of $CoCl_2$ by *t*-test. $^{*}P < 0.01$, significant difference between $CoCl_2$-treated PC12 cells in the absence or presence of scutellarin by *t*-test.

To assess intracellular ROS production, we measured the oxidation of the redox probe H_2DCFDA. After treatment with 500 μM $CoCl_2$ for 1 h, the ROS production increased significantly (Fig. 8). Preincubating with indicated amounts of scutellarin for 2 h decreased ROS production in a dose-dependent manner (Fig. 8).

Discussion

In the present study, we demonstrated that scutellarin (at dose of 0.1, 1 and 10 μM) significantly increased cell viability, decreased percentage of apoptosis population, improved morphological changes, inhibited caspase-3 activity, increased Bcl-X_L expression, reduced p38 MAPK phosphorylation and decreased ROS production in $CoCl_2$-treated PC12 cells. Therefore, the conclusion obtained from the above observations was that scutellarin can protect PC12 cells from apoptosis induced by $CoCl_2$.

The most characteristic traits of apoptosis are the fragmentation of nucleus with condensed chromatin and extensive membrane blebbing (28, 29). In our study, all of these morphologic features were observed in $CoCl_2$-treated PC12 cells. Scutellarin improved the morphological changes significantly. The increasing of cell viability and decreasing of percentage of apoptosis population confirmed the anti-apoptotic effect of scutellarin.

Caspase-3 is a key protease during apoptosis induced by various stimuli and is of particular importance in neurons (8, 17, 18, 24, 27). Prior studies showed that caspase-3 is activated in response to hypoxia (2, 3, 4, 14, 31) and caspase-3 is expressed in PC12 cells (12). Our previous studies have shown that caspase-3 is activated in $CoCl_2$-induced apoptosis in PC12 cells (33). In the present study, we found that the activity of caspase-3 was decreased after scutellarin treatment, which further confirmed the protective effect of scutellarin on PC12 cell apoptosis induced by $CoCl_2$.

Bcl-2 and Bcl-X_L are particularly important

molecular regulators in inhibiting apoptosis (20). A given cell type may express either or both of them. We failed to examine the expression of Bcl-2 in PC12 cells regardless of $CoCl_2$ treatment or not. We found that the expression of Bcl-X_L was down-regulated by $CoCl_2$, and scutellarin strongly increased its expression. Bcl-X_L acts as an inhibitor of apoptosis (25). The increase of Bcl-X_L after treatment of scutellarin may benefit better understanding of the mechanism of scutellarin in protecting neuronal apoptosis. It is well known that the over-expression of Bax can accelerate cell apoptosis triggered by a certain apoptotic stimuli. However, we failed to find any change of Bax in PC12 cells regardless of $CoCl_2$ treatment or scutellarin pre-incubation.

P38 MAPK has been demonstrated to be one of the apoptotic markers during PC12 apoptosis induced by various stimuli (11, 30). Both p38alpha and p38gamma are selectively activated during the hypoxia response in PC12 cells (7). The activation of p38 kinase may serve as a death signal, because addition of its inhibitor, SB203580, blocks caspase-3 activation and $CoCl_2$ induced apoptosis (33). Our present study showed that p38 MAPK was activated by $CoCl_2$ in PC12 cells and scutellarin, an antioxidant, inhibited the p38 MAPK activation, which is in agreement with the previous reports that ROS generation is upstream of p38 MAPK activation in the signaling cascade (16, 22).

ROS production has been shown to mediate apoptosis triggered by a wide range of influences including ultraviolet light, ionizing irradiation, anthracyclines, ceramides, glucocorticoids, TNF-α or survival-factor withdrawal (21). Studies have shown that ROS level is significantly increased in $CoCl_2$-induced PC12 cells and that RNA or protein synthesis inhibitors has no effects on ROS production regardless of $CoCl_2$ treatment or not, which suggest that ROS may serve as upstream mediators in apoptosis pathway (32). In the present study, we found that scutellarin strongly inhibited the ROS generation induced by $CoCl_2$. These results suggest that the antioxidative effect of scutellarin may be the mechanism of its protection on PC12 apoptosis induced by $CoCl_2$.

In conclusion, the present results show that scutellarin can protect PC12 cells from apoptosis induced by $CoCl_2$. The protection is accompanied by the alleviation of scutellarin on ROS production. These findings suggest that scutellarin is capable of protecting neurons from apoptotic cell death induced by hypoxia, which allows a better understanding regarding the potential clinical therapeutic use of scutellarin.

Acknowledgments

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