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Protective Effects of 20-Hydroxyecdysone on $CoCl_2$-Induced Cell Injury in PC12 Cells

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ABSTRACT

20-Hydroxyecdysone, which is found in the rhizomes, roots and the stems of many plants, is an ecdysteroid hormone that regulates molting in insects. We have previously shown that 20-Hydroxyecdysone could alleviate neurological deficits induced by subarachnoid hemorrhage in rabbits. Thus, we hypothesized that 20-Hydroxyecdysone might protect neurons against hypoxic–ischemic injury. In present study, the effects of 20-Hydroxyecdysone on cobalt chloride ($CoCl_2$)-induced cellular injury in PC12 cells was investigated. The incubation of PC12 cells with $CoCl_2$ reduced the cell viability, increased the rate of apoptosis. However, when cells were treated with 20-Hydroxyecdysone before or after $CoCl_2$ exposure, the $CoCl_2$-induced cellular injuries were significantly ameliorated. In addition, 20-Hydroxyecdysone dramatically reduced the $CoCl_2$-induced production of reactive oxygen species (ROS), decreased the depolarization of the mitochondrial membrane, inhibited the release of cytochrome *c* into the cytosol and increased the Bax/Bcl-2 ratio. Furthermore, 20-Hydroxyecdysone eliminated the $CoCl_2$-induced activation of caspase-3. Taken together, these results indicate that 20-Hydroxyecdysone may protect PC12 cells against $CoCl_2$-induced cell injury by inhibiting ROS production and modulating components of the mitochondrial apoptosis pathway. Based on our results, 20-Hydroxyecdysone may be a potential candidate for intervention in hypoxic–ischemic brain injuries such as stroke. J. Cell. Biochem. 111: 1512–1521, 2010.

KEY WORDS: 20-HYDROXYECDYSONE; $COCL_2$; PC12 CELLS; APOPTOSIS; REACTIVE OXYGEN SPECIES; MITOCHONDRIA

Cerebral hypoxia/ischemia is one of the most important and life-threatening causes of brain injury. The ischemic/hypoxic condition is accompanied by the production of reactive oxygen species (ROS) and other harmful chemical species [Cuzzocrea et al., 2001; Alexandrova et al., 2004]. Oxidative stress, which results from excessive cellular exposure to ROS is a fundamental mechanism of brain damage in ischemic/hypoxic conditions [Cuzzocrea et al., 2004; Cherubini et al., 2005]. In many cells, ROS causes DNA damage, protein oxidation and lipid peroxidation [Alexandrova et al., 2004; Esposito and Cuzzocrea, 2009]. In addition, intracellular ROS leads to the depolarization of the mitochondrial membrane, releasing mitochondrial cytochrome *c* into the cytosol and activating caspase, which subsequently contributes to apoptotic cell death [Zou et al., 2001; Jung et al., 2007; Wang et al., 2009a]. Preventing the production of ROS and regulating components of the mitochondria-mediated apoptotic pathway are thus primary goals in treating hypoxic/ischemic brain injuries such as stroke.

20-Hydroxyecdysone (Fig. 1A) is an insect steroid hormone belonging to the larger family of ecdysteroids and regulates molting, metamorphosis, and reproduction in arthropods [Briersa et al., 1983]. The hormone is found at higher concentration in plants, making it easier to extract 20-Hydroxyecdysone from plants than from arthropods. The acute toxicity of 20-Hydroxyecdysone is low in mice [Matsuda et al., 1970] and it has no side effects in humans and other mammals [Sláma and Lafont, 1995]. Although 20-Hydroxyecdysone does not bind to vertebrate steroid receptors and its mechanism of action is still unknown, the hormone displays a range of pharmacological properties such as, stimulating protein synthesis and promoting carbohydrate and lipid metabolism [Lafont and Dinan, 2003; Bathori and Pongracz, 2005; Oehme et al., 2006]. These effects explain why several ecdysteroid-containing plant species are used in traditional medicines. We have previously shown that 20-Hydroxyecdysone can alleviate neurological deficits that are induced by experimental subarachnoid hemorrhage in rabbits [Liu et al., 2008]. This result is consistent with the finding that 20-Hydroxyecdysone can attenuate vasospasm resulting from experimental subarachnoid hemorrhage [Tang et al., 2008]. However, it also possible that 20-Hydroxyecdysone has neuroprotective effects

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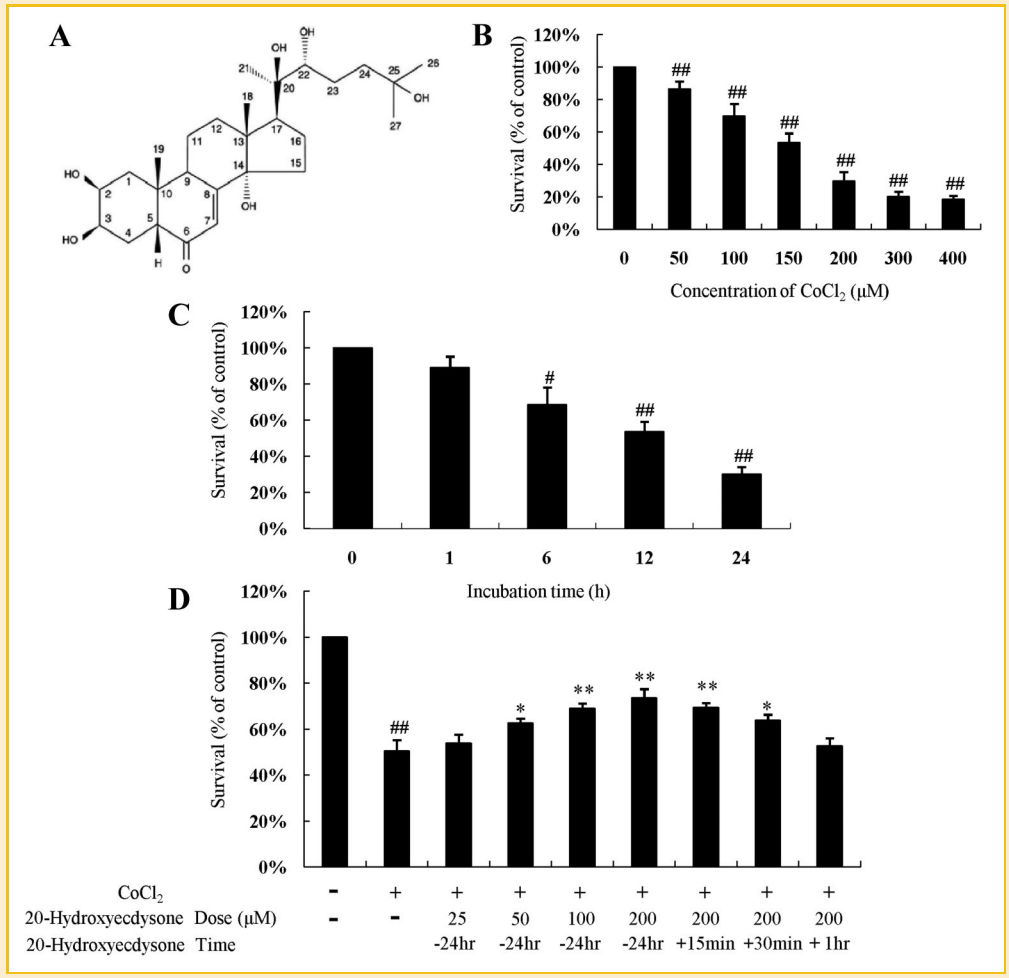

Fig. 1. Protective effects of 20-Hydroxyecdysone against $CoCl_2$ toxicity in PC12 cells. A: The chemical structure of 20-Hydroxyecdysone. B: Concentration-dependent effect of $CoCl_2$ on cell survival in PC12 cells. PC12 cells were exposed to different concentrations of $CoCl_2$ for 12 h. Cell viability was assessed using an MTT assay. C: Time course of cell death induced by 150 μM of $CoCl_2$. Cell viability decreased significantly 1 h after incubation with $CoCl_2$ and continued to decrease over the next 24 h. D: PC12 cells were treated with 150 μM $CoCl_2$ for 12 h. Some cells were pretreated with 25, 50, 100, or 200 μM 20-Hydroxyecdysone for 24 h prior to a 12 h incubation with $CoCl_2$ or treated with 200 μM 20-Hydroxyecdysone 15 min, 30 min, or 1 h after $CoCl_2$ exposure. Data are reported as means ± SD from three independent experiments. Non-treated cells served as controls. $^{\#}P < 0.05$ versus Control, $^{\#\#}P < 0.01$ versus Control, $^{*}P < 0.05$ versus $CoCl_2$ alone, $^{**}P < 0.01$ versus $CoCl_2$ alone. −24 or +1 h, 20-Hydroxyecdysone was administrated 24 h before or 1 h after $CoCl_2$ exposure; +15 or +30 min, 20-Hydroxyecdysone was administrated 15 or 30 min after $CoCl_2$ exposure.

because the neurological deficits induced by experimental subarachnoid hemorrhage are associated with hypoxic/ischemic neuronal damage. It has also been reported that 20-Hydroxyecdysone has anti-radical and anti-oxidative properties. In fact, its anti-oxidative activity equals known inhibitors of lipid peroxidation, such as diethyl *para*-phenylenediamine, and ethylenediaminetetraacetate [Kuz'menko et al., 1999]. In addition, 20-Hydroxyecdysone was more potent than vitamin D3, as demonstrated by studies analyzing blood serum as well as microsomal and mitochondrial fractions of rat liver [Kuzmenko et al., 1997]. Furthermore, several studies have shown that 20-Hydroxyecdysone protects neurons against the deleterious effects of diazepam and alcohol in mice [Xu et al., 1999]. Thus, we expect 20-Hydroxyecdysone to have beneficial effects in ischemic/hypoxic conditions that are associated with ROS production and neuronal apoptosis.

The rat pheochromocytoma cell line PC12 consists of catecholaminergic, excitable cells and has been widely used to study neuronal apoptosis in vitro [Wu et al., 2007; Jung et al., 2008]. $CoCl_2$, a water-soluble compound, was used in this investigation because it mimic hypoxic/ischemic conditions in various cultured cells [Zou et al., 2001; Li et al., 2008; Wang et al., 2009b]. Therefore, we used $CoCl_2$-treated PC12 cells as a model to study the neuronal response to hypoxia.

The primary purpose of this study was to determine whether 20-Hydroxyecdysone protects PC12 cells against $CoCl_2$-induced cell injury. In addition, we also examined the potential protective mechanisms induced by20-Hydroxyecdysone. We found that 20-Hydroxyecdysone exerts protective effects against $CoCl_2$-induced cell injury via its anti-oxidant and anti-apoptotic activity in PC12 cells.

MATERIALS AND METHODS

MATERIALS

Heat-inactivated horse serum, fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (NY).

Cobalt chloride ($CoCl_2$), Hoechst 33342, propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCF-DA) and Rhodamine 123 were purchased from Sigma Chemical Co. (MO). The in situ cell death detection kit was purchased from Roche (Mannheim, Germany). The annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit, Bicinchoninic Acid Kit for Protein Determination (BCA kit), anti-mouse IgG horseradish peroxidase (HRP) and anti-rabbit IgG-HRP were purchased from the Beyotime Institute of Biotechnology (Haimen, China). Cytochrome *c*, Bax, and Bcl-2 antibodies were purchased from Santa Cruz (Santa Cruz, CA). Enhanced chemiluminescence (ECL) was purchased from Pierce Chemical Company (Rockford, IL). The 20-Hydroxyecdysone (supplied by Shanghai Tauto Biotech Co., Ltd) was dissolved in ethanol and diluted with medium until use. For all experiments, the final concentration of ethanol was 0.1%, and control cultures received the carrier solvent (0.1% ethanol).

CELL CULTURE

Rat PC12 cells (adrenal gland; pheochromocytoma) were kindly provided by Yan Saia at the Department of Preventive Medicine, Third Military Medical University, Chongqing, China [Sai et al., 2008]. PC12 cells were kept at 37°C in DMEM containing 10% heat-inactivated horse serum and 5% fetal bovine serum in a water-saturated atmosphere with 5% CO_2. The culture medium was replenished at 3- to 4-day intervals based on the doubling time of PC12 cells. $CoCl_2$ was dissolved in distilled H_2O and sterilized through a 0.2 μm filter prior to use. Cells were kept in the same medium and treated with varying concentrations of $CoCl_2$ for the required time. Cells received 20-Hydroxyecdysone pretreatment for 24 h before $CoCl_2$ exposure or were treated 15 min, 30 min, or 1 h after $CoCl_2$ exposure.

MEASUREMENT OF CELL VIABILITY

Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals via mitochondrial dehydrogenases. Briefly, PC12 cells were plated at a density of 1×10^4 cells/well in 96-well plates and exposed to $CoCl_2$ alone. Some cells were pretreated with different concentrations of 20-Hydroxyecdysone (25, 50, 100, 200 μM) or treated with 200 μM 20-Hydroxyecdysone 15 min, 30 min, or 1 h after $CoCl_2$ exposure. After the treatments, MTT was added to the culture medium at a final concentration of 0.5 mg/ml and incubated at 37°C for 4 h. The reaction product of MTT was extracted in dimethylsulfoxide (DMSO) and the optical density (OD) was spectrophotometrically measured at 570 nm using an ELISA reader (Bio Tek Instruments, Winooski, VT) with DMSO as the blank. Cell viability was expressed as a percentage of the control.

CELL APOPTOSIS ASSESSMENT

Hoechst 33342 Staining. Morphological changes to the apoptotic cells were investigated using Hoechst 33342 staining. The cells were treated as described for the MTT assay and then fixed in 4.0% paraformaldehyde for 20 min and stained with 10 μg/ml Hoechst 33342 dye at 37°C for 10 min. The stained cells were washed twice with PBS and imaged using confocal laser microscopy (Leica TCS SP5, Wetzlar, Germany). The dye was excited at 340 nm, and the emission was filtered with a 510 nm barrier filter.

TUNEL Assay. The cells were treated as described above. The cells that had been fixed in 4.0% paraformaldehyde for 20 min were subjected to the TUNEL assay via an in situ cell death detection kit according to the manufacturer's specifications. The results were visualized using confocal laser microscopy (Leica TCS SP5). A standard fluorescein filter set was used to visualizing the green fluorescence of fluorescein at 520 ± 20 nm and the red fluorescence of PI at >620 nm. To quantify the apoptotic process, the cells with green fluorescence and the cells with red fluorescence were counted, respectively. Data are expressed as the ratio of TUNEL-positive cells to total cells.

Flow Cytometric Analysis. Flow cytometry was used to track membrane and nuclear events during apoptosis. The membrane events were analyzed by measuring the binding of FITC-Annexin V protein to the phospholipid phosphatidylserine that is present on the external surface of the apoptotic cell membrane. The assay was performed by a two-color analysis of FITC-labeled binding and PI uptake using the Annexin V-FITC Apoptosis Detection Kit. After positioning the quadrants on the Annexin V/PI dot plots, live cells (Annexin V−/PI−), early/primary apoptotic cells (Annexin V+/PI−), late/secondary apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V−/PI+) were distinguished [Wu et al., 2007]. When calculating the total percentage of cells with fluorescence Annexin V+/PI− and Annexin V+/PI+ were included. After treatment, the cells were trypsinized and centrifuged for 5 min. The supernatant was removed and 195 μl of binding buffer and 5 μl of Annexin V-FITC were added. The cells were incubated for 10 min in the dark at room temperature and then centrifuged. Following supernatant removal, 190 μl of binding buffer and 10 μl of PI were added to the cell pellet. The cells were then incubated for 5 min in the dark, at room temperature. The fluorescence of 10,000 events per sample was analyzed using flow cytometery (BD Immunocytometry Systems, San Jose, CA).

MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES FORMATION

The level of intracellular ROS was measured by using the fluorescent probe DCF-DA [Diehn et al., 2009; Wang et al., 2009a]. Intracellular H_2O_2 or low-molecular-weight peroxides oxidize DCF-DA to produce the highly fluorescent compound DCF. The cells were plated in 6-well plates and pretreated with either 5 mM *N*-acetyl-cysteine (NAC) for 1 h or 200 μM 20-Hydroxyecdysone for 24 h; both groups of cells were then treated with 150 μM $CoCl_2$ for 1 h. The treated cells were incubated with 30 μM DCF-DA for 30 min at 37°C and were then washed three times with PBS. Finally, Fluorescence intensity was monitored using flow cytometry (BD Immunocytometry Systems).

MEASUREMENT OF MITOCHONDRIAL TRANSMEMBRANE POTENTIAL

The mitochondrial membrane was monitored using the fluorescent dye Rhodamine 123, which preferentially partitions into active mitochondria based on the highly negative mitochondrial membrane potential. The depolarization of mitochondrial membrane results in the loss of Rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence [Chen et al., 2009]. PC12 cells

were either incubated with 5 mM NAC for 1 h or 200 μM 20-Hydroxyecdysone for 24 h before these cells are exposed to 150 μM $CoCl_2$ for 12 h. Rhodamine 123 (at a final concentration of 10 μM) was added to cells. After a 30-min culture at 37°C, the cells were collected, washed twice with PBS and analyzed by flow cytometry (BD Immunocytometry Systems).

WESTERN BLOT ANALYSIS

The cells were harvested following treatment. They were subsequently lysed in a buffer (50 mmol/L Tris–HCl, pH 8.0; 100 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L dithiothreitol; 1% Triton X-100; 0.1% sodium dodecyl sulfate; 50 mmol/L sodium fluoride, and 1 mmol/L sodium vanadate) containing a cocktail of protease inhibitors. The lysate was incubated on ice for 30 min and then centrifuged at 12,000*g* for 2 min at 4°C. The supernatant was collected and protein concentration was determined using a BCA kit. Equal amounts of protein were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, UK). The membrane was incubated in fresh blocking buffer (containing Tris-buffered saline, 0.1% Tween-20 in Tris-buffered saline, pH 7.4) at room temperature for 1 h and then incubated with anti-cytochrome *c* antibody, anti-Bcl-2 antibody, anti-Bax antibody or anti-cleaved caspase-3 overnight at 4°C. The blots were washed three times in TBS-T for 5 min and then incubated with specific peroxidase-coupled secondary antibodies (anti-mouse IgG-HRP or anti-rabbit IgG-HRP). The bound antibodies were visualized using an enhanced chemiluminescent detection system and then exposed to X-ray films (Kodak, USA). The images were scanned with a GS800 Densitometer Scanner (Bio-Rad, Hercules, CA), and OD data were analyzed using Quantity One software (Bio-Rad). In these analyses, β-actin was used as an internal reference.

STATISTICAL ANALYSIS

Data are expressed as means ± SD. The significance of inter-group difference was evaluated by one-way analyses of variance (ANOVA), and Duncan's test was used for post hoc comparisons. Differences were considered significant at $P < 0.05$ or $P < 0.01$.

RESULTS

20-HYDROXYECDYSONE ATTENUATED $COCL_2$-INDUCED CYTOTOXICITY IN PC12 CELLS

The MTT assay revealed that $CoCl_2$ decreased cell viability in a time- and concentration-dependent manner (Fig. 1B,C). The viability of cells treated with 150 μM $CoCl_2$ for 12 h was 53.6 ± 5.5% of the control value. Based on this result, a treatment of 150 μM $CoCl_2$ for 12 h was used to induce PC12 cell injury in subsequent experiments. As shown in Figure 1D, $CoCl_2$ (150 μM for 12 h) treatment decreased cell viability to 50.6 ± 4.7% of the control value. PC12 cells were then pretreated with a single dose of 20-Hydroxyecdysone 24 h before the onset of $CoCl_2$ treatment. Because no protection of PC12 cells was observed when 25 μM 20-Hydroxyecdysone was administrated, we therefore increased the amount of 20-Hydroxyecdysone to 50, 100, or 200 μM. The cell viability significantly increased to 62.6 ± 2.0% ($P < 0.05$ in comparison to the $CoCl_2$ treatment group), 68.9 ± 2.1% ($P < 0.01$ in comparison to the $CoCl_2$ treatment group) and 73.6 ± 3.8% ($P < 0.01$ in comparison to the $CoCl_2$ treatment group) of the control value at 50, 100, and 200 μM, respectively. We thus used 200 μM 20-Hydroxyecdysone in all subsequent experiments.

We next examined the time window of 20-Hydroxyecdysone protection. When 200 μM 20-Hydroxyecdysone was administered within 15 or 30 min after the onset of $CoCl_2$ exposure, the cell viability significantly increased to 69.4 ± 1.9% ($P < 0.01$ in comparison to the $CoCl_2$ treatment group) and 63.9 ± 2.4% ($P < 0.05$ in comparison to the $CoCl_2$ treatment group), respectively. However, 20-Hydroxyecdysone protection disappeared when it was administered 1 h after $CoCl_2$ exposure (Fig. 1D). In another experiment, a 48 h treatment with various concentrations (25–200 μM) of 20-Hydroxyecdysone alone did not cause any apparent increased in the viability of cells (data not shown). These findings demonstrate that 20-Hydroxyecdysone protects PC12 cells against $CoCl_2$-induced injury in a dose- and time-dependent manner.

20-HYDROXYECDYSONE PREVENTED $COCL_2$-INDUCED APOPTOSIS IN PC12 CELLS

Hoechst 33342 staining was used to evaluate the morphological changes that indicate apoptosis in PC12 cells. As shown in Figure 2A, the control cells that were not treated with $CoCl_2$ had round nuclei with uniformly dispersed chromatin. The cells treated with 150 μM $CoCl_2$ for 12 h displayed common characteristics of apoptosis, such as, the chromatin condensation, nucleus shrinkage and apoptotic bodies. However, a pretreatment with 200 μM 20-Hydroxyecdysone for 24 h before $CoCl_2$ exposure or a treatment with 200 μM 20-Hydroxyecdysone 15 min after $CoCl_2$ exposure markedly decreased the number of cells with nuclear condensation and fragmentation. Similarly, the TUNEL analysis showed that while control cells were generally TUNEL negative (7.4 ± 1.2%), $CoCl_2$ stimulation increased the percentage of TUNEL-positive cells (37.17 ± 5.2%, $P < 0.01$ in comparison to the control group). In contrast, when the cells were pretreated with 200 μM 20-Hydroxyecdysone for 24 h before $CoCl_2$ exposure or treated with 200 μM 20-Hydroxyecdysone 15 min after $CoCl_2$ exposure, the number of TUNEL-positive cells was significantly reduced to 20.31 ± 3.16% ($P < 0.05$ in comparison to the $CoCl_2$ treatment group) and 24.66 ± 4.98% ($P < 0.05$ in comparison to the $CoCl_2$ treatment group), respectively (Fig. 2B,C). Consistent with the TUNEL analysis data, the results of flow cytometric analysis further confirmed that 20-Hydroxyecdysone protected against $CoCl_2$-induced apoptosis in PC12 cells. As shown in Figure 3, the percentage of apoptotic PC12 cells increased from 5.33 ± 1.53% to 38.33 ± 3.51% after a challenge with $CoCl_2$. However, the percentage of apoptotic cells was significantly reduced to 18.67 ± 2.08% ($P < 0.01$ in comparison to the $CoCl_2$ treatment group) and 24.46 ± 4.83% ($P < 0.01$ in comparison to the $CoCl_2$ treatment group) when the cells were pretreated with 200 μM 20-Hydroxyecdysone for 24 h before $CoCl_2$ exposure or treated with 200 μM 20-Hydroxyecdysone 15 min after $CoCl_2$ exposure.

20-HYDROXYECDYSONE REDUCED $COCL_2$-INDUCED ROS GENERATION BY IN PC12 CELLS

The level of intracellular ROS significantly increased in PC12 cells after $CoCl_2$ exposure [Zou et al., 2001]. To examine whether the anti-

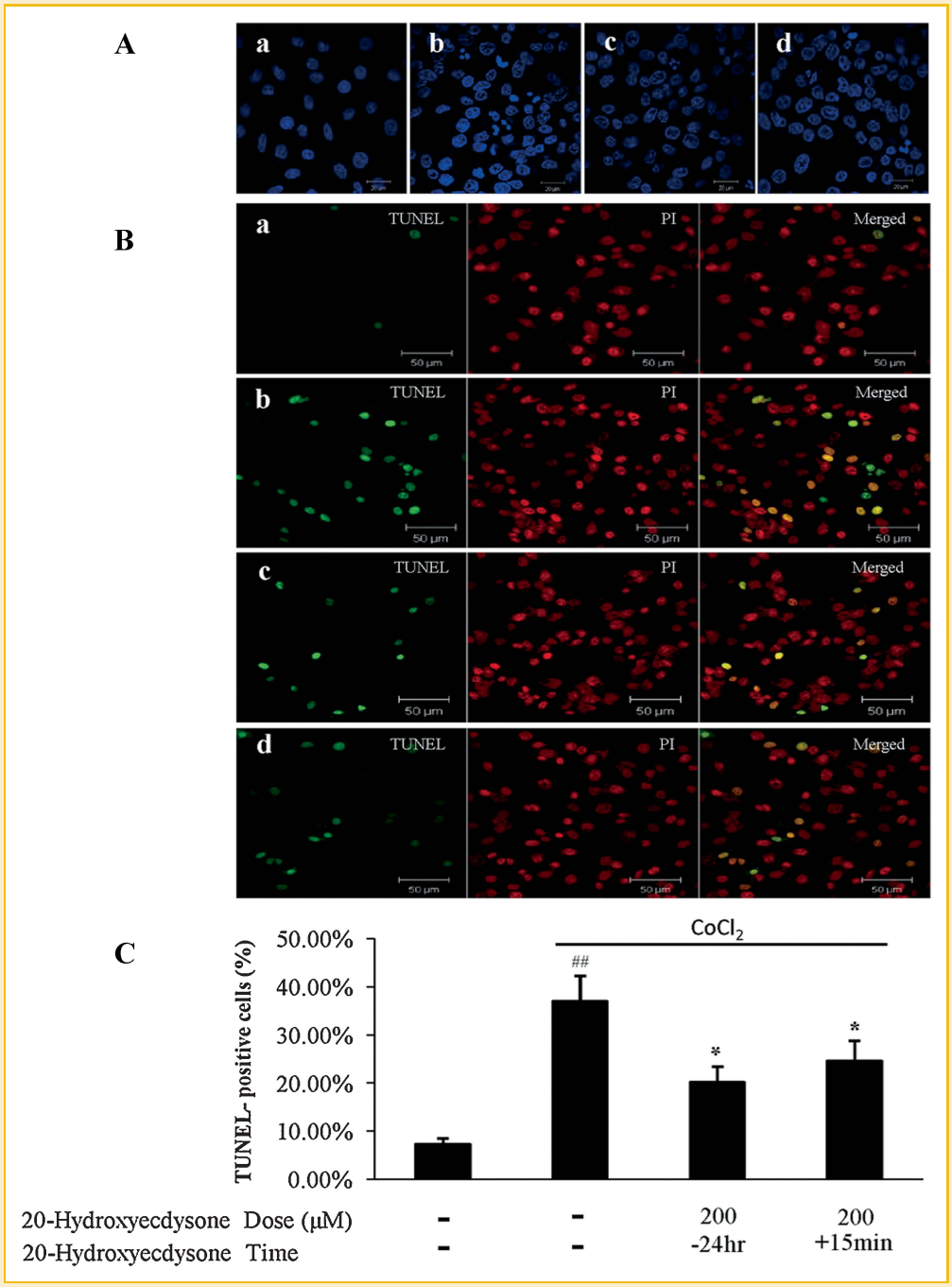

Fig. 2. Protective effects of 20-Hydroxyecdysone against $CoCl_2$-induced apoptosis in PC12 cells. The cells were incubated for 12 h with 150 μM $CoCl_2$ alone, pretreated with 200 μM 20-Hydroxyecdysone for 24 h before $CoCl_2$ exposure or treated with 200 μM 20-Hydroxyecdysone 15 min after $CoCl_2$ exposure. a: control; b: $CoCl_2$; c: 20-Hydroxyecdysone-pretreated for 24 h before $CoCl_2$ exposure; d: 20-Hydroxyecdysone-treated 15 min after $CoCl_2$ exposure. A: Morphological apoptosis was determined by Hoechst 33342 staining and confocal laser microscopy. Scale bar, 20 μm. B: Morphological apoptosis was examined by TUNEL analysis with TUNEL staining (green) and propidium iodide (PI, red) counterstaining. Images were obtained with confocal laser microscopy. Scale bar, 50 μm. C: Histogram showing the percentage of TUNEL-positive cells in the total cell population after different treatments. −24 h or +15 min, 20-Hydroxyecdysone was administrated 24 h before or15 min after $CoCl_2$ exposure. Data are reported as means ± SD from three independent experiments. Non-treated cells served as controls. $^{\#\#}P < 0.01$ versus Control, $^{*}P < 0.05$ versus $CoCl_2$ alone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

oxidative properties of 20-Hydroxyecdysone mediate its effect on $CoCl_2$ toxicity, PC12 cells were treated with 150 μM $CoCl_2$ for 1 h and the level of intracellular ROS was measured using flow cytometry with the molecular probe DCFH-DA. After the PC12 cells were treated, the intracellular DCF-fluorescence intensity significantly increased ($P < 0.01$ in comparison to the control group). However, pretreatment with either 200 μM 20-Hydroxyecdysone for 24 h or 5 mM NAC, ROS scavenger for 1 h before $CoCl_2$ exposure

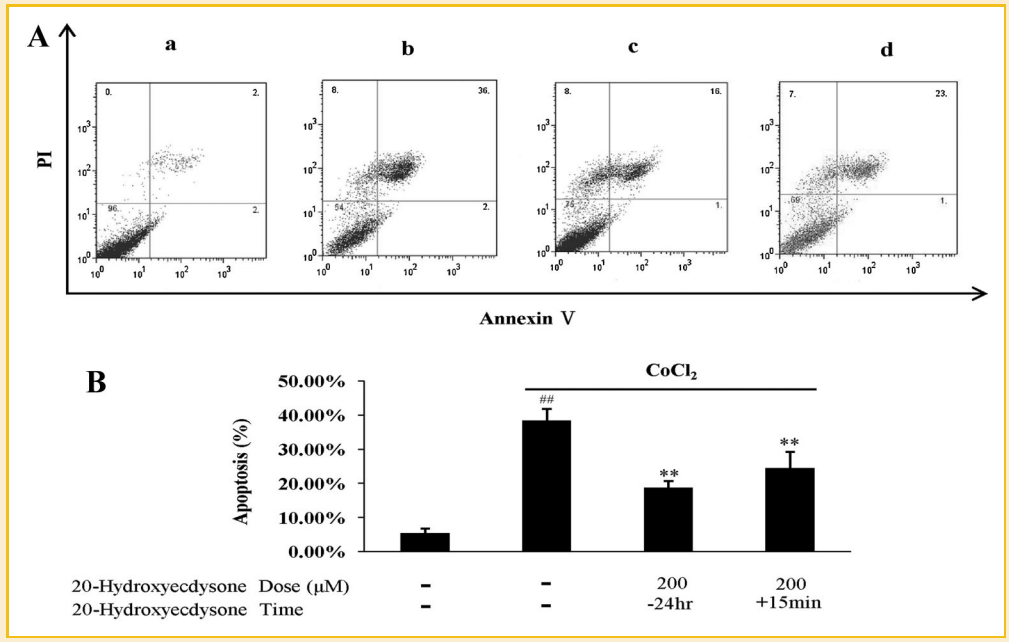

Fig. 3. Flow cytometric analysis of PC12 cells. Cells were treated as Figure 2. A: Apoptosis was determined by staining with Annexin-V + PI. a: control; b: $CoCl_2$; c: 20-Hydroxyecdysone-pretreated for 24 h before $CoCl_2$ exposure; d: 20-Hydroxyecdysone-treated 15 min after $CoCl_2$ exposure. B: Histogram showing the percentage of apoptotic cells and necrotic cells in the total cell population after different cell treatments. −24 h or +15 min, 20-Hydroxyecdysone was administrated 24 h before or15 min after $CoCl_2$ exposure. Data are reported as means ± SD from three independent experiments. Non-treated cells served as controls. $^{\#\#}P < 0.01$ versus Control, $^{**}P < 0.01$ versus $CoCl_2$ alone.

dramatically decreased the intracellular DCF-fluorescence intensity ($P < 0.01$ in comparison to the $CoCl_2$ treatment group). These results suggest that 20-Hydroxyecdysone inhibits $CoCl_2$-induced ROS production (Fig. 4).

20-HYDROXYECDYSONE RELIEVED MITOCHONDRIAL DEPOLARIZATION IN $COCL_2$-TREATED PC12 CELLS AND INHIBITED MITOCHONDRIAL CYTOCHROME *C* RELEASE INTO THE CYTOSOL

The depolarization of the mitochondrial membrane and the subsequent release of cytochrome *c* are characteristic features of early apoptosis and are induced by a variety of stimuli [Gogvadze et al., 2006]. The mitochondrial membrane potential in PC12 cells was determined using flow cytometry with the fluorescence probe Rhodamine 123. As shown in Figure 5A, no leakage of Rhodamine 123 was observed in untreated PC12 cells. After exposure to $CoCl_2$ for 12 h, the fluorescence intensity peak in $CoCl_2$-treated PC12 cells shifted to the left ($P < 0.01$ in comparison to the control group), indicating depolarization of the mitochondrial membrane. However, pretreatment with either 200 μM 20-Hydroxyecdysone for 24 h or 5 mM NAC for 1 h attenuated the shift caused by depolarization, increasing the fluorescence intensity of Rhodamine 123 ($P < 0.01$ in comparison to the $CoCl_2$ treatment group) (Fig. 5B). After the

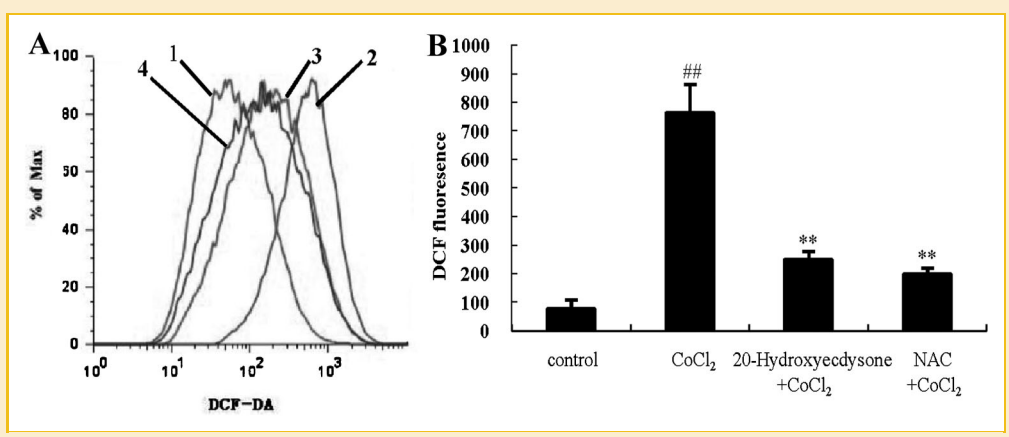

Fig. 4. Treatment with 20-Hydroxyecdysone prevented $CoCl_2$-induced oxidative stress. The DCF-loaded cells were incubated for 1 h with 150 μM $CoCl_2$ alone or pretreated with 200 μM 20-Hydroxyecdysone for 24 h or 5 mM NAC for 1 h. A: The DCF fluorescence was measured by flow cytometry. 1: Control; 2: $CoCl_2$; 3:20-Hydroxyecdysone + $CoCl_2$; 4: NAC + $CoCl_2$. B: Histogram showing the intracellular fluorescence intensity of DCF in different treatment cells. Data are reported as means ± SD from three independent experiments. Non-treated cells served as controls. $^{\#\#}P < 0.01$ versus Control, $^{**}P < 0.01$ versus $CoCl_2$ alone.

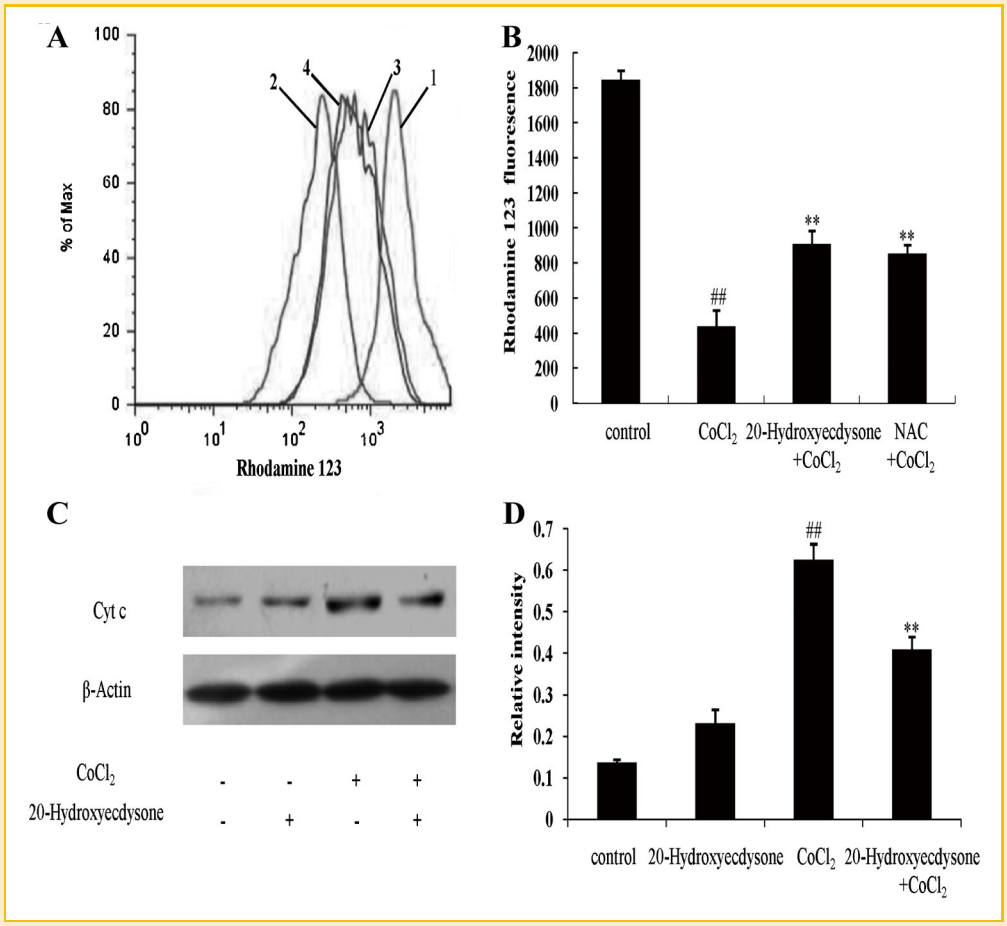

Fig. 5. Treatment with 20-Hydroxyecdysone prevented the $CoCl_2$-induced depolarization of the mitochondrial membrane and the release of mitochondrial cytochrome *c* into the cytosol in PC12 cells. A: The cells were incubated for 12 h with 150 μM $CoCl_2$ alone or pretreated with 200 μM 20-Hydroxyecdysone for 24 h or 5 mM NAC for 1 h. After cells were collected and stained with Rhodamine 123, Rhodamine 123 uptake was measured by flow cytometry. 1: Control; 2: $CoCl_2$; 3:20-Hydroxyecdysone + $CoCl_2$; 4: NAC + $CoCl_2$. B: Histogram showing the mean relative fluorescent density of Rhodamine 123 in different treatment cells. C: PC12 cells were treated with 150 μM $CoCl_2$ for 12 h. Some cells were pretreated with 200 μM 20-Hydroxyecdysone for 24 h prior to a 12 h incubation with 150 μM $CoCl_2$ or treated with 200 μM 20-Hydroxyecdysone alone for 24 h. Cytosolic cytochrome *c* levels were determined by Western blot analyses. Representative image of immunoblots for cytosolic cytochrome *c*. D: Densitometric analysis of changes to cytosolic cytochrome *c* levels. Data are reported as means ± SD from three independent experiments. Non-treated cells served as controls. $^{\#\#}P < 0.01$ versus Control, $^{**}P < 0.01$ versus $CoCl_2$ alone.

depolarization of mitochondrial membrane, cytochrome *c* is released into cytosol and subsequently cleaves pro-caspase-3 to form active caspase-3 [Yang and Cortopassi, 1998]. Western blot analysis revealed that $CoCl_2$ caused cytochrome *c* to accumulate in the cytosol ($P < 0.01$ in comparison to the control group). When the cells were pretreated with 200 μM 20-Hydroxyecdysone prior to $CoCl_2$ treatment, cytochrome *c* in the cytosol was significantly reduced when compared to the $CoCl_2$-treated group ($P < 0.01$) (Fig. 5C,D).

EFFECTS OF 20-HYDROXYECDYSONE ON THE $COCL_2$-INDUCED EXPRESSION OF THE BCL-2 FAMILY IN PC12 CELLS

The members of the Bcl-2 family are important regulators of mitochondrial integrity, mitochondria-initiated cytochrome *c* release and caspase activation. Both Bax and Bcl-2 belong to the Bcl-2 family. Bax is a pro-apoptotic protein that could regulates of cytochrome *c* release from mitochondria under a variety of stress conditions, whereas Bcl-2 is an anti-apoptotic protein that prevents cytochrome *c* release by heterodimerizing with Bax. The balance between these proteins is critical in activating and deactivating the cellular apoptotic machinery [Cory and Adams, 2002; Borner, 2003]. In this study, we used western blot analysis to investigate whether 20-Hydroxyecdysone has any effect on Bax and Bcl-2 expression in $CoCl_2$-treated cells. As shown in Figure 6, Bax protein expression increased significantly in $CoCl_2$-treated group compared with that in the control group ($P < 0.05$). However, 20-Hydroxyecdysone pretreatment could decrease the Bax expression level ($P < 0.05$) and increase the Bcl-2 expression level compared with that of the $CoCl_2$ treatment group ($P < 0.01$). After the treatment of PC12 cells for 24 h with 20-Hydroxyecdysone at 200 μM, the protein levels of both Bcl-2 and Bax were similar to that of the control group. The Bax/Bcl-2 ratio increased to 1.7-fold of the control group ($P < 0.01$) following treatment with $CoCl_2$, whereas 20-Hydroxyecdysone pretreatment prevented the $CoCl_2$-induced increase of the Bax/Bcl-2 ratio ($P < 0.01$ in comparison to the $CoCl_2$ treatment group).

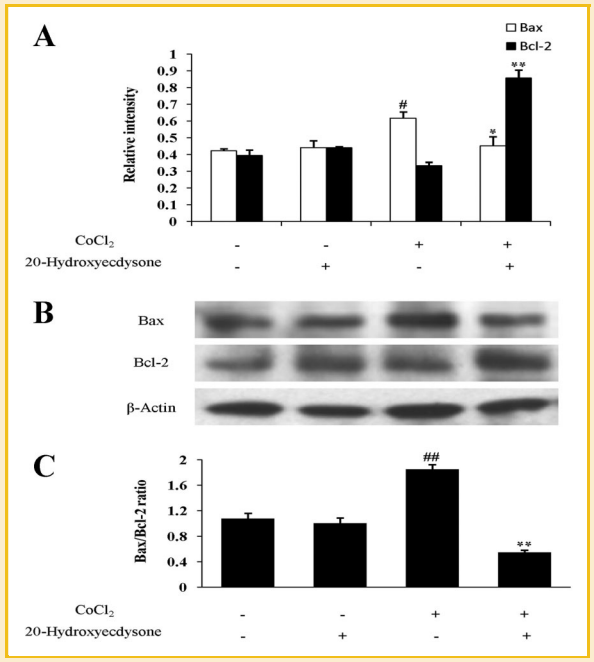

Fig. 6. Effect of 20-Hydroxyecdysone on Bax and Bcl-2 expression in $CoCl_2$-induced PC12 cells. PC12 cells were treated with 150 μM $CoCl_2$ for 12 h. Some cells were pretreated with 200 μM 20-Hydroxyecdysone for 24 h prior to a 12 h incubation with 150 μM $CoCl_2$ or treated with 200 μM 20-Hydroxyecdysone alone for 24 h. Bcl-2 and Bax protein levels in PC12 cells were measured by Western blotting. A: Densitometric analysis of changes to Bax and Bcl-2 levels. B: Representative image of immunoblots for Bax and Bcl-2. C: Effect of 20-Hydroxyecdysone on the Bax/Bcl-2 ratio. Data are reported as means ± SD from three independent experiments. Non-treated cells served as controls. $^{\#}P < 0.05$ versus Control, $^{\#\#}P < 0.01$ versus Control, $^{*}P < 0.05$ versus $CoCl_2$ alone, $^{**}P < 0.01$ versus $CoCl_2$ alone.

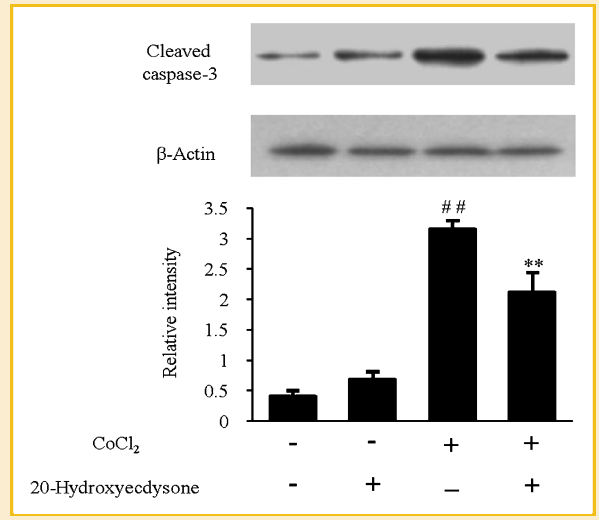

Fig. 7. 20-Hydroxyecdysone inhibited the $CoCl_2$-induced increase of cleaved caspase-3 protein levels in PC12 cells. PC12 cells were treated with 150 μM $CoCl_2$ for 12 h. Some cells were pretreated with 200 μM 20-Hydroxyecdysone for 24 h prior to a 12 h incubation with 150 μM $CoCl_2$ or treated with 200 μM 20-Hydroxyecdysone alone for 24 h. Cleaved caspase-3 levels were determined using Western blot analysis. A: Representative image of immunoblots for cleaved caspase-3. B: Densitometric analysis of changes to cleaved caspase-3 levels. Data are reported as means ± SD from three independent experiments. Non-treated cells served as controls. $^{\#\#}P < 0.01$ versus Control, $^{**}P < 0.01$ versus $CoCl_2$ alone.

20-HYDROXYECDYSONE PREVENTED THE ACTIVATION OF CASPASE-3 IN $COCL_2$-TREATED PC12 CELLS

Caspase-3 is thought to be a primary effector of apoptosis in response to diverse stimuli. Its activation leads to DNA breakage, nuclear chromatin condensation, and cell apoptosis [Talanian et al., 1997]. To investigate whether the prevention of $CoCl_2$-induced apoptosis by 20-Hydroxyecdysone depends on caspase-3 activation, cleaved caspase-3 levels were measured using Western blot analysis. As shown in Figure 7, cleaved caspase-3 expression was increased after exposure to $CoCl_2$ relative to the control group ($P < 0.01$), but the level of activated caspase-3 protein declined in the group pretreated with 20-Hydroxyecdysone ($P < 0.01$ in comparison to the $CoCl_2$ treatment group).

DISCUSSION

Hypoxia/ischemia-induced cell death becomes a major concern following brain injury. In this study, we used $CoCl_2$-induced insult in PC12 cells to partially simulate the pathological process of cerebral ischemia, attempting to search for a naturally occurring drug with neuroprotective effects. *Achyranthes bidentata* has been used in the treatment of trauma, thrombosis, and arthritis in traditional Chinese medicine for more than 1,800 years. The main active monomer component of *Achyranthes bidentata* is 20-Hydroxyecdysone. Previous studies have shown that 20-Hydroxyecdysone has anti-radical and anti-oxidative properties, exerting protective effects after drug-induced cerebral injury in animal models [Kuzmenko et al., 1997; Kuz'menko et al., 1999]. We have also shown that 20-Hydroxyecdysone alleviates neurological deficits and vasospasm resulting from experimental subarachnoid hemorrhage in rabbits [Liu et al., 2008; Tang et al., 2008]. Here, we investigated the effects of 20-Hydroxyecdysone on $CoCl_2$-induced cell death in PC12 cells, hoping to gain new insight into the neuroprotective effects of 20-Hydroxyecdysone in hypoxia/ischemia.

The present study showed that 20-Hydroxyecdysone has protective effects against PC12 cells injury in $CoCl_2$-induced hypoxia/ischemia. The protective roles of 20-Hydroxyecdysone were investigated using MTT assay measurements, Hoechst 33342 staining (a DNA-binding dye), TUNEL assay and flow cytometric analysis. Our results revealed that 20-Hydroxyecdysone prevented the $CoCl_2$-induced reduction in cell viability. In the cellular ultrastructure, 20-Hydroxyecdysone blocked the morphological changes caused by $CoCl_2$-induced apoptosis. Similarly, the flow cytometric analysis showed that 20-Hydroxyecdysone decreased the apoptotic rate throughout the experiment.

In subsequent experiments, we explored the mechanisms underlying the protective effects of 20-Hydroxyecdysone in $CoCl_2$-induced injury to PC12 cells. Our results demonstrated that several mechanisms, either working separately or in concert, may be involved in the

protective effects of 20-Hydroxyecdysone. Most importantly, 20-Hydroxyecdysone may directly scavenge ROS produced by $CoCl_2$. Several studies have shown that $CoCl_2$ treatment leads to the formation of intracellular ROS [Zou et al., 2001; Chen et al., 2009]. The production of ROS can lead to several events that play critical roles in apoptosis, including DNA cleavage, protein damage, and cell membrane lipid destruction [Floyd and Carney, 1992; Love, 1999; Murin et al., 2001]. Furthermore, ROS may also trigger apoptosis via mitochondria-mediated pathway because cell membrane lipid destruction could be partially responsible for the depolarization of mitochondrial membrane [Orrenius, 2007]. In addition, 20-Hydroxyecdysone has anti-radical properties because it can react with radicals by abstracting hydrogen from carbon 9 at B-C ring junction [Cai et al., 2002; Krishnan et al., 2007]. In this study, the $CoCl_2$ induced ROS formation in PC12 cells was inhibited by 20-Hydroxyecdysone to a similar degree as NAC, a ROS scavenger. These findings indicate that the anti-oxidative properties of 20-Hydroxyecdysone may contribute to the protection of PC12 cells from $CoCl_2$ damage.Mitochondria play a critical role in the mitochondrial apoptosis pathway [Green and Reed, 1998; Wang, 2001]. The depolarization the mitochondrial membrane results in a rapid release of caspase activators such as cytochrome *c* [Cai et al., 1998]. The release of cytochrome *c* from mitochondria into the cytosol is essential for caspase activation, which triggers downstream cell death pathways [Shi, 2002; Boatright and Salvesen, 2003]. In the present study, we found that $CoCl_2$ can depolarize the mitochondrial membrane and increased the release of mitochondrial cytochrome *c* into the cytosol. This finding suggests that mitochondrial signaling plays a key role in $CoCl_2$-induced apoptosis. Interestingly, 20-Hydroxyecdysone attenuated the depolarization of mitochondrial membrane and reduced the release of cytochrome *c*. This results support that 20-Hydroxyecdysone may exert some of its protective effects by modulating the mitochondrial apoptosis pathway.

The Bcl-2 family of anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL) and pro-apoptotic proteins (e.g., Bax and Bid) are important modulators of the mitochondrial apoptosis pathway [Korsmeyer, 1992; Oltvai et al., 1993]. Bcl-2 prevents the depolarization of the mitochondrial membrane and the production of ROS by mitochondria, thus preventing apoptosis [Burlacu, 2003; Tsujimoto, 2003]. In contrast, Bax induces mitochondrial depolarization and increases the production of ROS by mitochondria [Brady and Gil-Gomez, 1998]. Alterations in the ratio of pro- and anti-apoptotic Bcl-2 family proteins play a significant role in determining whether apoptosis occurs [Korsmeyer et al., 1993; Delchev et al., 2006]. In this study, we showed that $CoCl_2$ results in a significant decrease in Bcl-2 protein levels and an increase in Bax protein levels, thus shifting the Bax/Bcl-2 ratio in favor of apoptosis. However, these changes were attenuated by a pretreatment with 20-Hydroxyecdysone. This result indicates that 20-Hydroxyecdysone inhibits $CoCl_2$-induced apoptosis via the regulation of Bcl-2/Bax expression.

Caspases play a critical role in apoptosis induced by hypoxia/ischemia [Ho et al., 2006]. Caspase-3 is an effector caspase found in apoptotic cells. The activation of caspase-3 may be important for initiating or executing neuronal apoptosis [Nath et al., 1998]. Our results showed that $CoCl_2$ increased the activation of caspase-3 in PC12 cells. However, we also demonstrated that 20-Hydroxyecdysone effectively suppressed the $CoCl_2$-induced activation of caspase-3, suggesting that 20-Hydroxyecdysone may act upstream of caspase-3 to block apoptosis. Based on our own observations, the mechanisms by which 20-Hydroxyecdysone inhibits the $CoCl_2$-triggered activation of caspase-3 may include both its anti-oxidative activity and its ability to regulate the Bcl-2 family.

At present, many neuroprotective agents have proved to be strongly neuroprotective in laboratory models but failed in all possible clinical trials. Obviously positive outcomes in laboratory models do not guarantee success in clinical trials. On the other hand, a positive outcome in laboratory models is a prerequisite for continuing a development program into the clinical phase. This study demonstrates that 20-Hydroxyecdysone protects against $CoCl_2$-induced PC12 cell injury via its anti-oxidative effects and its ability to block the release of mitochondrial cytochrome *c* into the cytosol, modulating the ratio of Bax to Bcl-2 and attenuating the $CoCl_2$-induced activation of caspase-3. In addition, the acute toxicity of 20-Hydroxyecdysone is low in mice [Matsuda et al., 1970] and it has no side effects in humans and other mammals [Sláma and Lafont, 1995]. These features of 20-Hydroxyecdysone warrant further study of its protective effect for possible clinical use.

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