Protective effects of edaravone against cobalt chloride-induced apoptosis in PC12 cells

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Abstract: Objective To investigate the neuroprotective effects of edaravone (Eda) on cobalt chloride (CoCl_2)-induced oxidative stress and apoptosis in cultured PC12 cells as well as the underlying mechanisms. **Methods** PC12 cells impaired by CoCl_2 were used as the cell model of hypoxia. MTT (methyl thiazolyl tetrazolium) was used to assay the viability of the PC12 cells exposed to Eda with gradient concentrations; Hoechst 33258 stain assay was used to analyze the apoptosis ratio of the PC12 cells; Bcl-2 and Bax protein levels in PC12 cells were examined by western blotting. ROS level, the mitochondrial transmembrane potential and caspase-3 activity in each group were detected by spectrofluorometer. **Results** CoCl_2 treatment caused the loss of cell viability in PC12 cells, which was associated with the elevation of apoptotic rate, the formation of ROS and the disruption of mitochondrial transmembrane potential. CoCl_2 also significantly induced the upregulation of Bax/Bcl-2 ratio and the activation of caspase-3. In contrast, Eda significantly reversed these phenotypes, with its maximum protective effect at $0.1 \mu\text{mol/L}$. **Conclusion** These results indicated that Eda could protect PC12 cells from CoCl_2 -induced cytotoxicity, and this protection might be ascribed to its anti-oxidative and anti-apoptotic activities.

Keywords: CoCl_2 ; PC12 cells; edaravone; oxidative stress; apoptosis

1 Introduction

It has been identified that apoptosis may be involved in neuronal diseases relevant to hypoxia^[1]. Hypoxic/ischemic condition has long been recognized as an important mediator or modulator of apoptosis in neuronal cells because this condition is accompanied by the production of reactive oxygen species (ROS) which can attack nucleic acids, proteins and membrane phospholipids^[2,3]. The distinct mechanisms that execute apoptosis through various apoptotic stimuli, are ultimately classified into the mitochondria-dependent path-

way (intrinsic pathway) and the death receptor-dependent pathway (extrinsic pathway)^[4]. The mitochondria-dependent apoptotic pathway is stimulated under hypoxic/ischemia conditions^[5]. These stimuli induce generation of ROS, loss of mitochondrial transmembrane potential ($\Delta\psi\text{m}$), release of cytochrome c from the impaired mitochondria to cytosol. The released cytochrome c forms a complex with Apaf-1 in the presence of dATP, which recruits and activates caspase-9. Activated caspase-9 induces the activation of caspase-3, which subsequently contributes to apoptotic cell death^[6, 7].

Several reports indicated that cobalt chloride (CoCl_2) could mimic the hypoxic/ischemic conditions including the production of ROS in various cultured cells^[8-10]. PC12 is a cell line derived from rat pheochromocytoma and has been widely used as an *in vitro* model for investigating neuronal apoptosis, oxygen sensor mechanism, and neuronal differentiation^[11]. Therefore, we used CoCl_2 -treated PC12 cells as

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a model to study the neuronal cell response to hypoxia.

Edaravone (Eda) is kind of free-radical scavenger that has been evaluated as a neuroprotective compound which inhibits the increase of hydroxyl radical and superoxide anion level in several models of cerebral ischemia^[12,13]. Based on the fact that CoCl_2 induces a hypoxic/ischemic condition, this study was designed to investigate in molecular level the neuroprotective effects of Eda on pathways involved in CoCl_2 -induced apoptosis.

2 Materials and methods

2.1 Materials Eda was purchased from Mitsubishi Chemical Industries (Japan). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 2', 7'-dichlorofluorescein diacetate (DCF-DA), rhodamine 123 were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was from Biosea BCL (Beijing, China). The *in situ* cell death detection kit was from the Boehringer Mannheim, Co. (Mannheim, Germany). The caspase-3 fluorescent assay kit was from R&D systems (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated calf serum and fetal bovine serum were purchased from Gibco-BRL-Life Technologies (Gaithersburg, MD). Bicinchoninic Acid Kit for protein determination (BCA kit) and Enhanced chemiluminescence (ECL) were purchased from Pierce Chemical Company (Rockford, IL, USA).

2.2 Cell culture Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Chinese Type Culture Collection. PC12 cells were cultured in DMEM supplemented with 10% heat inactivated calf serum, 5% fetal bovine serum, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin in a water-saturated atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every three days and cells were subcultured about once a week. At 24 h before experiments, the medium was substituted by serum-deprived medium and cells were washed with serum-free DMEM and replanted in the 96 and 24 well plates. The next day CoCl_2 and/or Eda were added to naive PC12 cells.

2.3 Cell viability PC12 cells were seeded in 96-well plates at a density of 4×10^4 cells per well and were treated with various

concentrations of Eda (0.001, 0.01, 0.1, 1 and 10 $\mu\text{mol}/\text{L}$) and/or CoCl_2 at 37 °C. After incubation for up to 8 h, 10 μL of 5 mg/mL MTT solution was added and incubated for 4 h. Then the culture medium with MTT was removed and 200 μL dimethyl sulphoxide (DMSO) was added to each well for formazan dissolution. Absorbance was measured at 570 nm (540 nm as a reference) with a model 550-microplate reader. Cell viability was expressed as the percentage of the value in control cultures.

2.4 Hoechst 33258 staining Cells were washed with PBS, and fixed with a mixture of acetic acid-ethanol (1:3) for 10 min at room temperature, and then dropped on slide glasses. After being air dried, cells were washed and stained with 1 mg/mL Hoechst 33258 for 10 min at room temperature. The chromatin structures of the cells were examined by fluorescence microscopy.

2.5 Measurement of ROS generation DCF, the nonfluorescent cell-permeant compound that is intracellularly cleaved by endogenous esterases and is retained by viable cells, is often used as an indicator of ROS, on the finding that oxidation of DCF by ROS produced the fluorescent compound 29, 79-dichlorofluorescein (Bass *et al.*, 1983). To measure the ROS, PC12 cells were harvested and preincubated with 10 mmol/L DCF for 30 min in PBS containing 5 mmol/L glucose at 37 °C. After washing twice, cells were resuspended with PBS containing 5 mmol/L glucose and incubated at 37 °C for 30 min with indicated inducers. Fluorescence intensity was detected by spectrofluorometer (Hitachi F4010), with an excitation wavelength at 488 nm and an emission wavelength at 525 nm.

2.6 Measurement of mitochondrial transmembrane potential Mitochondrial transmembrane potential was measured using the dye rhodamine 123, since the uptake of rhodamine 123 into mitochondria was shown to be a function of mitochondrial transmembrane potential. PC12 cells (4×10^4) were incubated with Eda and/or CoCl_2 , followed by 10 $\mu\text{mol}/\text{L}$ rhodamine 123 (in DMEM) incubation for 20 min at 37 °C. Cell suspension was centrifugated at 412 g for 10 min, and precipitates were dissolved with 1% Triton X-100. Fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 510 nm using a fluorescence

microplate reader.

2.7 Western blot analysis Cells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1.55% DTT). Cell lysates (30 µg of protein) were loaded and separated by SDS-PAGE (12% for Bax, Bcl-2 and Bcl-XL) and transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL). Immunodetection was carried out using appropriate antibodies and proteins were visualized with enhanced chemiluminescence (ECL) system (Amersham).

2.8 Measurement of caspase-3 activity Caspase-3 activity was detected using the caspase-3 fluorometric assay kit (R&D) according to manufacturer's instructions. Synthetic tetrapeptide DEVD-7-amino-4-trifluoromethyl coumarin (AFC) was used as the substrate in this assay. Briefly, active caspase-3 induced the cleavage between DEVD and AFC sites of the substrate, releasing the fluorogenic AFC, which could be detected by spectrofluorometry. Thus, the fluorogenic AFC reflected the activity of caspase-3. After the incubation with Eda and/or CoCl₂, cells were collected and lysed in lysis buffer on ice for 10 min. The protein concentrations of the supernatant fluids were ascertained with the BCA kit. 200 µg protein was mixed with the reaction buffer and DEVD-AFC substrate, followed by 2 h incubation at 37 °C. The fluorescence was measured at an excitation wavelength of 400 nm and emission wavelength of 505 nm with fluorometric reader. A caspase-3-like activity was expressed as relative content against that in the cells incubated in the medium containing serum without CoCl₂.

2.9 Statistical analysis Data were presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Eda inhibited CoCl₂-induced cell viability loss in PC12 cells To assess the effect of Eda itself on cell viability of PC12 cells, cells were exposed to Eda with varying concentrations from 0.001 to 10 µmol/L for 8 h. The MTT assay revealed that Eda itself did not induce any loss of cell viability

(Fig. 1A).

However, CoCl₂ treatment at different doses (30, 75, 150, 300 and 600 µmol/L) for 8 h caused a loss of viability in a dose-dependent manner (Fig. 1B). Cell viability after 150 µmol/L CoCl₂ treatment for 8 h was only about 52.9±4.8% of the control value.

Whereas the Eda treatment at a concentration from

Fig. 1 Effects of Eda and CoCl₂ on the viability of PC12 cells. A: Eda did not show any toxicity on PC12 cells; B: CoCl₂ reduced the cell viability in a dose-dependent manner; C: After incubation in the media containing 150 µmol/L CoCl₂ and Eda (0, 0.001, 0.01, 0.1, 1 and 10 µmol/L) for 8 h, CoCl₂ induced cell death was rescued by Eda in a concentration-dependent manner. Data were presented as mean ± SD from triplicate independent experiments. ## $P < 0.01$ vs. the untreated control, * $P < 0.05$ and ** $P < 0.01$ vs. the CoCl₂-treated group.

Fig. 2 PC12 cells stained with Hoechst 33258. After the cells were incubated with 150 $\mu\text{mol/L}$ CoCl_2 and/or with 0.1 $\mu\text{mol/L}$ Eda for 8 h, nuclear condensation in the apoptotic cell was analyzed by fluorescent staining with Hoechst 33258. A: Control; B: 150 $\mu\text{mol/L}$ CoCl_2 treated alone; C: 150 $\mu\text{mol/L}$ CoCl_2 with 0.1 $\mu\text{mol/L}$ Eda; D: 0.1 $\mu\text{mol/L}$ Eda treated alone. The figures are representative for three different experiments. Scale bar, 10 μm .

0.001 to 10 $\mu\text{mol/L}$ prevented the viability loss (Fig. 1C). After the incubation with both Eda (0.1 $\mu\text{mol/L}$) and CoCl_2 (150 $\mu\text{mol/L}$), the cell viability increased to $64.7 \pm 5.2\%$.

The results in Fig. 1 indicated that Eda exhibited neuroprotective effects on CoCl_2 -induced cytotoxicity in PC12 cells in a dose-dependent manner, with its optimal concentration at 0.1 $\mu\text{mol/L}$.

3.2 Eda rescued CoCl_2 -induced changes in nuclear morphology To assess CoCl_2 induced apoptotic cell death and clarify the inhibitory effect of Eda on the cytotoxicity of CoCl_2 , we investigated the effect of Eda on the nuclear morphological changes in CoCl_2 -treated cells. Nuclear staining with Hoechst 33258 demonstrated that control PC12 cells had regular and round-shaped nuclei. In contrast, condensation and fragmentation of nuclei, the characteristics of apoptotic cells, were evident in cells treated with 150 $\mu\text{mol/L}$ CoCl_2 (Fig. 2). Eda (0.1 $\mu\text{mol/L}$) ameliorated the CoCl_2 -induced nuclear damage, while Eda alone did not change the nuclear morphology compared with the control cells.

3.3 Eda attenuated CoCl_2 -induced increase in intracellular

Fig. 3 Eda reduced CoCl_2 -induced increase in intracellular ROS concentration in PC12 cells. The intracellular levels of ROS were detected by measuring the DCF fluorescence. Eda attenuated an increase in ROS level induced by CoCl_2 at 150 $\mu\text{mol/L}$. ## $P < 0.01$ vs control; ** $P < 0.01$ vs CoCl_2 group (CoCl_2 at 150 $\mu\text{mol/L}$).

ROS level As shown in Fig. 3, when PC12 cells were exposed to 150 $\mu\text{mol/L}$ CoCl_2 for 8 h, the intracellular reactive oxygen species level (mean fluorescent intensity, MFI) significantly increased from $2522.6 \pm 265\%$ (control) to $5865.2 \pm 475.5\%$ ($P < 0.01$), revealing that CoCl_2 enhanced reactive oxygen species concentration in PC12 cells. However, treatment with Eda effectively reduced reactive oxygen species generation,

and the suppressing effect strengthened with the increase of Eda concentration. Eda inhibited the 150 $\mu\text{mol/L}$ CoCl_2 -induced increase in DCF fluorescence, with a maximum inhibition at 0.1 $\mu\text{mol/L}$.

3.4 Eda ameliorated the mitochondrial transmembrane potential (MMP) loss caused by CoCl_2 In this respect, we assessed the effect of CoCl_2 on the mitochondrial transmembrane potential. Change in the mitochondrial transmembrane potential in PC12 cells caused by CoCl_2 was quantified by measuring the cellular retention of rhodamine 123. Exposure to CoCl_2 at 150 $\mu\text{mol/L}$ for 8 h considerably reduced the mean fluorescent intensity (MFI) of rhodamine 123 stain from 0.479 ± 0.0392 (control) to 0.102 ± 0.0276 ($P < 0.01$), indicating a fall in the MMP and an inhibitory effect of CoCl_2 on MMP (Fig. 4). However, when PC12 cells were co-treated with Eda (0.1 $\mu\text{mol/L}$) and CoCl_2 (150 $\mu\text{mol/L}$), the MFI was significantly enhanced to 0.397 ± 0.032 ($P < 0.01$), suggesting that Eda inhibited the fall of MMP induced by CoCl_2 (150 $\mu\text{mol/L}$), and at 0.1 $\mu\text{mol/L}$, Eda showed a maximum inhibition (Fig. 4).

3.5 Eda attenuated the CoCl_2 induced increase of Bax to Bcl-2 ratio To determine whether Eda protects CoCl_2 -induced apoptosis of PC12 cells by modulating the Bcl-2 family, protein levels of Bax and Bcl-2 were observed using western blot analysis. As shown in Fig. 5, CoCl_2 increased the Bax expression level and decreased the Bcl-2 expression, while 0.1 $\mu\text{mol/L}$ Eda treatment inhibited upregulation of Bax and enhanced upregulation of Bcl-2 slightly. Therefore, Eda attenuated the increase in Bax to Bcl-2 ratio caused by CoCl_2 ,

Fig. 4 Eda blocked the CoCl_2 -induced reduction of mitochondrial membrane potential (MMP). The MMP was assessed by the mean fluorescent intensity (MFI) of rhodamine 123 staining in PC12 cells. ### $P < 0.01$ vs control; *** $P < 0.01$ vs CoCl_2 (150 $\mu\text{mol/L}$) group.

Fig. 5 Effects of Eda on the expression of the Bcl-2 family. A: After the cells were incubated with 150 $\mu\text{mol/L}$ CoCl_2 or with 100 $\mu\text{mol/L}$ Eda 1 h prior to treatment for 8 h, western blot was performed for Bax and Bcl-2 expression. B: The Bax and Bcl-2 ratio determined by a densitometer was then calculated and graphed. Data were presented as mean $\pm$ SD from triplicate independent experiments. # $P < 0.05$ vs control; ** $P < 0.05$ vs CoCl_2 (150 $\mu\text{mol/L}$) group.

which was a sign of apoptosis inhibition (Fig. 5).

3.6 Eda inhibited caspase-3 activity in PC12 cells induced by CoCl_2 After the 150 $\mu\text{mol/L}$ CoCl_2 treatment for 8 h in PC12 cells, we detected an increase of caspase-3 like activity compared with the control cells. In contrast, PC12 cells which were simultaneously treated with Eda (0.001, 0.01, 0.1, 1, 10 $\mu\text{mol/L}$) showed a significant decrease in caspase-3 activity compared with the CoCl_2 -treated cells [(0.397 $\pm$ 0.032)%,

Fig. 6 Eda inhibited CoCl_2 -induced increase in caspase-3 activity. Values represent 3 replicate values in 4 separate experiments (means $\pm$ SD). * $P < 0.05$, ** $P < 0.01$ vs CoCl_2 alone; ## $P < 0.01$ vs control.

(0.303 ± 0.029)%, (0.165 ± 0.023)%, (0.254 ± 0.034)%, (0.351 ± 0.035)%, respectively, Fig. 6]. Eda alone did not show a significant effect on the caspase-3 activity in PC12 cells. The results showed that Eda treatment resulted in the inhibition of CoCl_2 -induced activation of caspase-3, with a maximum inhibition at $0.1 \mu\text{mol/L}$ concentration.

4 Discussion

Hypoxia-induced cell death is a major concern in various clinical fields such as ischemic disease, organ transplantation, and other diseases. CoCl_2 -induced cell death in PC12 cells may serve as a simple and convenient *in vitro* model of hypoxia-induced neuronal cytotoxicity to elucidate molecular mechanisms underlying the hypoxia-linked cell death and to search possible treatments since CoCl_2 mimics hypoxic/ischemic conditions including ROS production in neuronal cells. Previous studies have reported that the signal pathways of CoCl_2 -induced apoptosis of PC12 cells were involved with mitochondria-mediated pathway^[9]. The main mechanism for apoptosis appeared to be mitochondria-mediated pathway, which usually accompanied with loss of the $\Delta\psi\text{m}$ followed by cytochrome *c* release from the mitochondria into the cytosol, resulting in the activation of caspase-9 and caspase-3. Also, there occurred significant upregulation of pro-apoptotic protein Bax and downregulation of anti-apoptotic protein Bcl-2 in the presence of CoCl_2 , which then might result in activation of mitochondria-mediated apoptosis.

Some reports showed Eda could protect neural system against ischemia or hypoxia challenge. Using a rat model of transient middle cerebral artery occlusion, researchers concluded that daily treatment with Eda helped reduce cortical infarction volume^[14]. Eda also salvaged the boundary zone of infarct by scavenging reactive oxygen species, especially in the neurons during permanent focal cerebral ischemia^[15]. In addition, Eda not only had antioxidant roles, but also protected cells from apoptosis^[16,17]. However, little information is available on antioxidant Eda in regulating the Bcl-2/Bax apoptotic pathway via mitochondria after CoCl_2 injury at cellular level.

In present study, we first observed the effects of CoCl_2 on PC12 cells and found that CoCl_2 impaired the cell viability

and induced apoptosis in PC12 cells. To evaluate Eda as neuroprotective agent in this paper, the protective roles of Eda were investigated by MTT assay and Hoechts 33258 staining. According to these results, Eda could exert protective effects against CoCl_2 -induced cell viability loss. Eda also blocked apoptosis in morphological changes, which showed that Eda decreased the apoptotic rate during the whole experimental process.

The present study shows that Eda exerts the neuroprotective effect in a concentration dependent manner against the CoCl_2 induced cell viability loss in PC12 cells. The neuroprotective effect of Eda reached maximum at $0.1 \mu\text{mol/L}$, while it obviously decreased at larger doses. The higher concentration ($10 \mu\text{mol/L}$) of Eda did not show protective effect. Further experiments are still required to identify the optimal dose regimen for clinical application.

However, up to date, the mechanisms that underlie the protective effect of Eda on the neurotoxicity of CoCl_2 have not been fully understood. Subsequent experiments were designed to explore the mechanisms of the neuroprotection of Eda on CoCl_2 -induced cell death in PC12 cells. Here, our results demonstrate that several mechanisms, separately or in association, may be involved in the neuroprotective effects of Eda. A body of work has been generated to support the premise that CoCl_2 can directly lead to the formation of ROS and mitochondrial dysfunction^[9,18]. The production of ROS can lead to cell injury through cell membrane lipid destruction and DNA cleavage^[19,20], thus plays a critical role in leading to final apoptosis. Recent investigations indicated that mitochondria played a prominent role in transduction and amplification of the apoptotic response. Eda has been shown to afford its neuroprotection by its antioxidant effects^[21,22], which is further confirmed by our data that Eda is highly effective in inhibiting CoCl_2 -induced ROS formation and mitochondrial membrane potential loss in PC12 cells. The present results suggest that Eda attenuates the mitochondrial damage and cell death in PC12 cells caused by oxidative stress. Based on these findings, we postulate that the anti-oxidative properties of Eda may contribute to the protection of PC12 cells from CoCl_2 damage.

Consequently, some other mechanisms could also be

pertinent in the Eda protective mechanism. Bcl-2 family plays a key role in the mitochondrial apoptotic pathway^[23]. In the present study, Eda decreased the ratio of Bax to Bcl-2, by blocking the CoCl₂-induced downregulation of Bcl-2. These results demonstrate that Eda inhibits CoCl₂-induced apoptosis through the regulation of the Bcl-2 family. Furthermore, Eda attenuated the CoCl₂-induced increase in caspase-3 activity. From our own observations, a decrease in caspase-3 activity correlates well with a decrease in the Bax/Bcl-2 ratio, as pro-apoptotic Bax is thought to be upstream of the caspase in the mitochondria-mediated apoptotic death pathway^[23], suggesting that the mechanisms by which Eda inhibits CoCl₂-triggered activation of caspase-3 might include both its anti-oxidative activity and its regulatory function in Bcl-2 family. These results indicate that the neuroprotective effects of Eda may result from the inactivation of caspase cascade associated with mitochondria pathway by modulating Bcl-2 family on CoCl₂-mediated apoptosis.

In this study, we demonstrated that Eda possessed a significant neuroprotective role against CoCl₂-induced apoptosis in PC12 cells. Eda ameliorated CoCl₂-induced ROS production, attenuated the mitochondrial transmembrane potential loss, downregulated the Bax/Bcl-2 ratio, and also prevented the activation of caspase-3. These findings support the theory that cytoprotection mediated by Eda is due, in part, to inhibition of the oxidative stress resulting from the mitochondrial apoptotic pathway.

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依达拉奉对氯化钴诱导的 PC12 细胞凋亡的保护作用

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摘要: **目的** 探讨依达拉奉(edaravone, Eda)对氯化钴(CoCl₂)诱导的PC12细胞凋亡的保护作用及其机制。**方法** 用CoCl₂处理PC12细胞建立细胞缺氧模型,采用四甲基偶氮唑蓝法检测暴露于不同浓度Eda后细胞的活性;Hoechst 33258染色检测各组的细胞凋亡状况;免疫印迹法检测不同处理组PC12细胞Bcl-2和Bax的表达,并采用荧光法观察不同处理组PC12细胞活性氧(reactive oxygen species, ROS)与线粒体膜电位水平以及caspase-3活性的变化。**结果** CoCl₂可以使PC12细胞存活率下降,凋亡率增高;同时PC12细胞内ROS增多,线粒体膜电位下降。CoCl₂还可以明显地提高Bax/Bcl-2比值并激活caspase-3。而Eda可以抑制这些由CoCl₂引发的改变,并在0.1 μmol/L发挥最大保护作用。**结论** Eda可抑制CoCl₂诱导的PC12细胞死亡,其作用机制可能与其自身抗氧化、抗凋亡的特性有关。

关键词: 氯化钴; PC12 细胞; 依达拉奉; 氧化应激; 凋亡