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Bcl-2 Family and Caspases are Involved in $CoCl_2$-Induced Apoptosis of PC12 Cells

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Hypoxic/ischemic condition induces the neuronal apoptotic events, consequently resulting in neuronal damages. Cobalt chloride ($CoCl_2$) could mimic the hypoxic condition including the production of reactive oxygen species (ROS). This study aimed to investigate the roles of Bcl-2 family and caspases as central regulators of apoptosis, in $CoCl_2$-induced apoptosis of PC12 cells.

Cell viability was determined by MTT assay and DNA fragmentation was detected by DNA laddering. The expression levels of Bcl-2, Bax, Bid, cytochrome *c* and Fas/APO-1 were determined by RT-PCR or Western blotting analysis in $CoCl_2$-treated PC12 cells. Caspase-9 and caspase-3 activities were assessed using spectrophotometry and caspase-8 activity was measured with fluorospectrocytometry.

Administration of $CoCl_2$ decreased viability of cells in a dose- and time-dependent manner. Furthermore, fragmentation of the genomic DNA and apoptotic bodies were induced in $CoCl_2$-treated PC12 cells. Bcl-2, an anti-apoptotic Bcl-2 family, was downregulated, whereas Bax, pro-apoptotic molecule, was upregulated in $CoCl_2$-treated cells. Treatment of $CoCl_2$ augumented the release of cytochrome *c* into the cytoplasm and increase of caspase-8, -9, and -3 activities. In addition, $CoCl_2$ upregulated Fas and downregulated pro-Bid, which are known to be correlated with death receptor-mediated apoptotic signaling pathway.

Therefore, these results suggest that Bcl-2 family and caspase play crucial roles in $CoCl_2$-induced apoptosis through mitochondria- and death receptor-dependent pathways in PC12 cells.

Key words : PC12 cells, $CoCl_2$, Apoptosis, Caspase, Bcl-2 family, Mitochondria

Introduction

Apoptosis, a gene-regulated mechanism of cell death, is involved in the control of cell number and the removal of inappropriate or damaged cells (Adrens et al. 1990). Hypoxic/ischemic condition has long been recognized as important modulators of apoptosis because this condition is accompanied by the production of reactive oxygen species (ROS) which can attack nucleic acids, proteins and membrane phospholipids (Zhang and Wang 1999, Wang et al. 2000, Cao et al. 2001). Hypoxia/ischemia-induced apoptosis in neuronal cells is a major concern in various clinical entities such as ischemic disease, organ transplantation and other disease. However, the apoptotic mechanisms of neuronal cells in hypoxic/ischemic condition remained unsettled yet.

In general, apoptosis is activated by caspases, a family of cysteine protease, which then cleave a critical set of cellular proteins to induce apoptotic cell death (Roth et al. 2000, Crompton 2000). These family are

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expressed as proenzymes and are activated by upstream stimuli. Among mammalian caspases of at least 14 known members, those involved with apoptosis can be further subdivided into the initiator caspases (-2, -8, -9, and -10) and the effector caspases (-3, -6, and -7) (Adams and Cory 1998, Tsujimoto and Shimizu 2000). Two main pathways of activating caspases are death receptor-mediated and mitochondria-mediated mechanisms. Both pathways share the activation of caspase-3 as an executioner caspase, which activates caspase-activated DNase, causing apoptotic DNA fragmentation. Death receptor pathway is stimulated by cell surface death receptors such as tumor necrosis factor receptor and Fas/APO-1 (Beer et al. 2000). The receptors activated by ligands lead to caspase-8 activation, with subsequent activation of caspase-3. The mitochondrial pathway is stimulated by hypoxic/ischemic condition, cytotoxic reagents, radiation, and growth factor deprivation (Zou et al. 2001, 2002). These stimuli induce release of cytochrome *c* from mitochondria into cytosol, subsequently resulting in caspase-9 activation which causes the activation of caspase-3. However, the death receptor-mediated and mitochondria-mediated apoptotic mechanism of neuronal cells in hypoxic/ischemic condition has been little studied. Besides the caspase, members of the Bcl-2 family are also critical for the regulation of apoptosis. Bcl-2 family control the release of mitochondrial cytochrome *c* by regulating the permeability of the outer mitochondrial membrane. Bcl-2 family members are functionally divided into anti-apoptotic molecules (Bcl-2, Bcl-X_L, Bcl-W and Mcl-1) and pro-apoptotic molecules (Bax, Bid, Bad, Bim and Bik) (Adams and Cory 1998, Tsujimoto and Shimizu 2000). Among the Bcl-2 protein family, Bcl-2 and Bcl-X_L are prominent anti-apoptotic family, whereas Bax and Bid are prominent pro-apoptotic family (Cheng et al. 1997). However, the roles of Bcl-2 family in hypoxic/ischemic-induced neuronal apoptosis also have not been still elucidated.

Cobalt chloride ($CoCl_2$) can mimic hypoxic/ischemic conditions, including the generation of ROS and transcriptional change of some genes such as p53, p21 and pCNA in promoting the cell death (Adams and Cory 1998, Thornberry and Lazabnik 1998, Earnshaw et al. 1999, Strasser et al. 2000). PC12 is a cell line derived from rat pheochromocytoma widely used for investigating neuronal apoptosis (Walkinshaw and Waters 1994). Therefore, $CoCl_2$-induced apoptosis may serve as a simple and convenient *in vitro* model to elucidate molecular mechanism in hypoxia-induced neuronal cell death and to search its treatment methods.

The present study was designed to investigate the roles of Bcl-2 family and caspases associated with mitochondria- and death receptor-mediated apoptotic pathway in $CoCl_2$-treated PC12 cells as model of neuronal hypoxic/ischemic conditions.

Materials and Methods

1. Cell culture and cell viability assay

PC12 rat adrenal pheochromocytoma cells were maintained in RPMI 1640 medium supplemented with 10% horse serum (Gibco BRL, Rockville, USA) and 5% fetal bovine serum (Gibco BRL) under 5% CO_2 at 37°C. $CoCl_2$ was dissolved in distilled H_2O and sterilized through 0.2 μm filter. To examine cell viability, the cells were incubated in RPMI 1640 medium with low serum (only 10% FBS), and then were determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra zolium bromide) assay (Sigma, St. Louis, USA).

2. Detection of ROS production

ROS production was monitered by fluoresence spectrophotometer (Hitachi F-4500, Tokyo, Japan) using DCF-DA (2′, 7′-dichlorofluorescin diacetate). Cells were plated on 48-well plates and treated with NAC (N-acetyl-cystein) and $CoCl_2$. DCF-DA (25 μM)

was added into the midium for further 15 min at 37℃. Emission was measured at 530 nm.

3. Nuclear staining with propidium iodide (PI)

Morphological changes of apoptotic cells were investigated by PI. Cells were plated in 8-well chamber slides at a density of 1×10^5 and incubated for 18 h, subsequently followed by treatment with 150 μM $CoCl_2$ for 12 h. The cells were then washed with PBS and fixed with acetone and methanol (1 : 1). After incubating for 20 min at −20℃, cells were stained with 10 μg/mL of PI in PBS and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

4. Agarose gel electrophoresis for DNA fragmentation

Oligonucleosomal fragmentation of genomic DNA was assessed using the Apopladder kit (TaKaRa Shuzo, Tokyo, Japan) according to the manufacturer's instructions. Briefly, cells were lysed with 200 μL of lysis buffer and centrifuged at 1,100 × g for 10 min. The supernatant was then incubated at 56℃ for 1 h after adding 20 μL of 10% SDS and 20 μL of proteinase K (20 mg/mL), and then treated with 1 μg of RNase at 37℃ for 1 h. DNA was extracted and precipitated overnight at −20℃ in a precipitant mixture containing 0.95 mL of ethanol and pelleted by centrifugation for 15 min at 10,000 × g at 4℃. DNA pellets were resuspended in 20 μL of TE (pH 8.0) and aliquots from each sample were electrophoresed at 80 V for 2 h on 2.0% agarose gels.

5. Analysis of caspase activity

PC12 cells were grown on 60 mm dishes at a concentration of 5×10^5 cells and treated with 150 μM $CoCl_2$ for 12 h or 24 h. Caspase activities were assayed using the caspase-3 and caspase-9 activity assay kits (Calbiochem, La Jolla, CA) and a caspase-8 activity kit (Santa Cruz, California, USA) according to the manufacturer's instructions.

6. Reverse transcription polymerase chain reaction (RT-PCR)

For extraction of total RNA, cells were homogenized with a polytron homogenizer in Trizol reagent (Gibco-BRL, Rockville, USA). For synthesis of cDNA, 1 μg of total RNA and 1 μL of Oligo (dT) (10 pmoles) were mixed with 50 μL RNase-free water, and then incubated at 42℃ for 1 h and 94℃ for 5 min. PCR products were generated in PCR buffer containing 10 pmoles of each primer using PCR-premix kit (Bioneer, Seoul, Korea). After the first denaturation step (5 min at 95℃), samples were subjected to 30 cycles consisting of 40 sec at 95℃, 40 sec at 55℃, and 1 min 30 sec at 72℃, with a final extention step of 10 min, on a GeneAmp PCR system (Perkin-Elmer 2400, Boston, USA). The following primer pairs were used: for Bax, 5′-GTTTCATCCAGGATCGAGCAG-3′(senseprimer) and 5′-CATCTTCTTCCAGATGGTGA-3′ (antisense primer); for Bcl-2, 5′-CCTGTGGATGACTGGTACC-3′(sense primer) and 5′-GAGACAGCCAGGAGAAATCA-3′(antisense primer). The amplified products were analyzed on 1.5% agarose gels containing ethidium bromide and visualized by UVP Transilluminator/Polaroid camera System (UVP Laboratories, CA, USA). RT-PCR was performed with primers for the housekeeping gene, GAPDH, as a control. The following primer pairs for GAPDH were used: 5′-TGCATCCTGCACCACCAACT-3′(sense primer) and 5′-CGCCTGCTTCACCACCTTC-3′(antisense primer). The intensities of the obtained bands were determined using the NIH Scion Image Software.

7. Western blot analysis

Cells were washed twice with PBS and proteins solubilized in the lysis buffer (500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzamiden,

1 μg/mL trypsin inhibitor) containing a cocktail of protease inhibitor (Complete, Boehringer Mannheim, Germany). To determinate cytosolic cytochrome *c*, pellet was resuspended in extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-NaOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, and 1 mM DTT. Lysates were incubated for 30 min at 4℃, centrifuged at 11,000 × g for 20 min and protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). Protein extracts (100 ~ 500 μg) were boiled for 5 min with SDS-sample buffer and then subjected to electrophoresis on 12% polyacrylamide gel. Proteins were electroblotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, NJ, USA) and blocked with 5% skim milk (Becton Dickinson, NJ, USA) in Tris-buffered saline-0.1%Tween 20 (TBS-T). Primary antibodies used were rat monoclonal anti-cytochrome *c* (Pharmingen, Sandiego, CA), Fas/APO-1 (Pharmingen), β-Actin (Santa Cruz, California, CA) and Bid (Santa Cruz) were applied. Blots were subsequently washed three times in TBS-T for 5 min and incubated with specific peroxidase-coupled secondary antibodies (Sigma). Bound antibodies were visualized using an enhanced chemiluminescent detection system (Amersham Pharmacia Biotech, NJ, USA).

Results

1. $CoCl_2$ induces ROS production and apoptosis in PC12 cells

To determine the role of ROS in $CoCl_2$-induced apoptosis, ROS production was measured in the $CoCl_2$ -treated cells using the fluorophore DCF-DA. Fig. 1 showed that $CoCl_2$ induced the ROS production. Pretreatment of cells with 5 mM NAC, a ROS scavenger, inhibited the constitutive and $CoCl_2$-induced ROS. The cell viability was determined by MTT assay. As

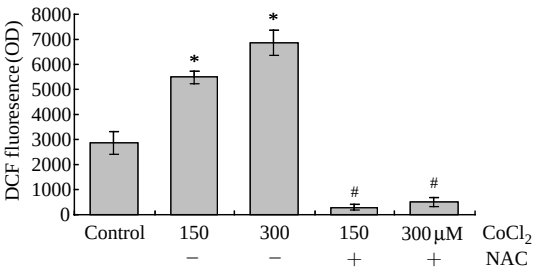

Fig. 1. ROS production was enhanced in $CoCl_2$-treated PC12 cells. DCF-loaded cells were incubated with 150 μM $CoCl_2$ alone or co-incubation with 5 mM NAC for 12 h. The intracellular levels of ROS were detected by measuring the DCF fluorescence. Data are mean ± SD from 5 independent experiments. $^{*}p < 0.05$ versus the corresponding control value, $^{\#}p < 0.01$ versus the corresponding 150 μM $CoCl_2$-treated cells.

shown in Fig. 2, treatment of $CoCl_2$ greatly reduced the viability of PC12 cells in a dose and time-dependent manner. The cell survival was less than 60% when the cells were treated with 150 μM $CoCl_2$ for 12 h. In the presence of 150 μM $CoCl_2$ for 24 h, PI staining revealed apoptotic morphological changes, including chromatin condensation and nuclear fragmentation (Fig. 3A). DNA was isolated from $CoCl_2$-treated PC12 cells and then the degradation of nuclear DNA into oligonucleosome fragments was assessed. The DNA laddering pattern was detectable in $CoCl_2$-treated PC12 cells (Fig. 3B). These results demonstrate that cell death by $CoCl_2$ in PC12 cells occurs via apoptosis.

2. Bax is upregulated and Bcl-2 is downregulated in $CoCl_2$-induced apoptosis

Generally, expression ratio of Bax to Bcl-2 has proven to be a significant factor for apoptosis determination. After the treatment of PC12 cells with 150 μM $CoCl_2$ for 12 h or 24 h, the changes in the mRNA expression levels of Bax and Bcl-2 in PC12 cells treated with $CoCl_2$ were determined by RT-PCR. $CoCl_2$

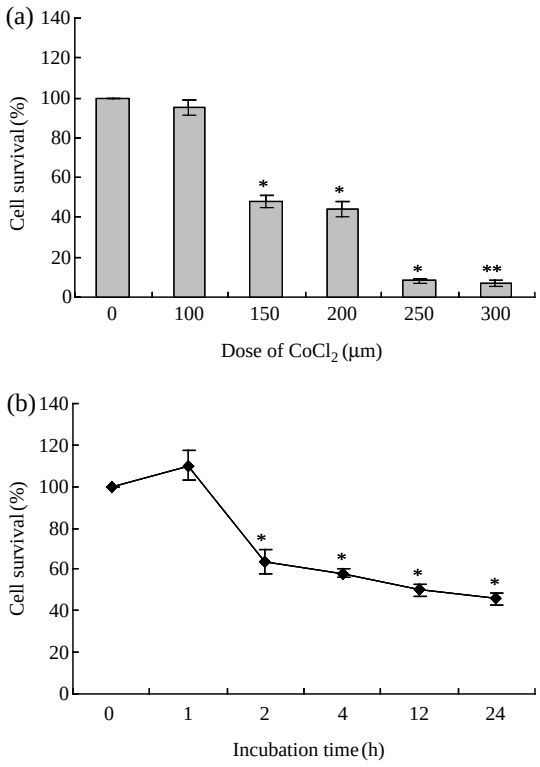

Fig. 2. $CoCl_2$ induced cell death in PC12 cells. PC12 cells were incubated with different $CoCl_2$ doses for 12 h (a) and with 150 μM $CoCl_2$ for indicated times (b). Viability of the cells without $CoCl_2$ was defined as 100%. Data are mean ±SD from 5 independent experiments. The viability was reduced in time- and dose-dependent manners in $CoCl_2$-treated PC12 cells. *$p < 0.05$ and **$p < 0.01$ versus the corresponding control value.

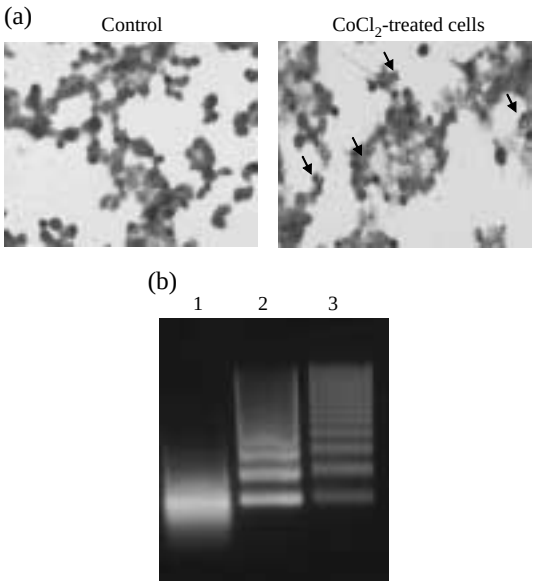

Fig. 3. $CoCl_2$ induced morphologic changes and DNA fragmentation in PC12 cells. (a) Cells were treated with $CoCl_2$ for 24 h and fixed with ethanol and DNA was stained using PI to show chromatin condensation. (b) Cells were incubated in the absence(lane 1), or in the presence of 150 (lane 2) and 200 (lane 3) μM $CoCl_2$ for 24 h.

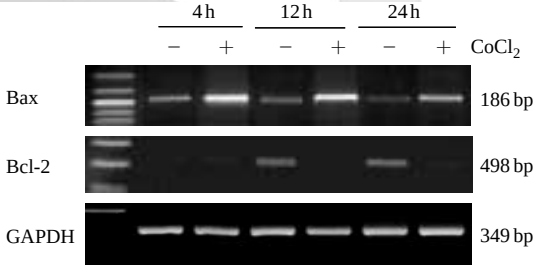

Fig. 4. Expression of Bax was upregulated and Bcl-2 was downregulated in $CoCl_2$-treated PC12 cells. After incubation of cells with 150 μM $CoCl_2$ for indicated time, RT-PCR was performed for Bax and Bcl-2 expression.

induced the Bcl-2 expression and repressed the Bax expression (Fig. 4).

3. $CoCl_2$ induced the release of cytochrome *c* from mitochondria to cytoplasm

Cytoplasmic concentration of cytochrome *c* were assessed as a consequence of cytochrome *c* released from mitochondria into cytoplasm. PC12 cells were incubated with various concentrations of $CoCl_2$ for different periods, and subjected to Western blot analysis. The release of cytoplasmic cytochrome *c* was enhanced in dose-dependent manner upto 150 μM $CoCl_2$, but slightly decreased at concentration of 200 μM (Fig. 5A). Cytosolic cytochrome *c* reached peak

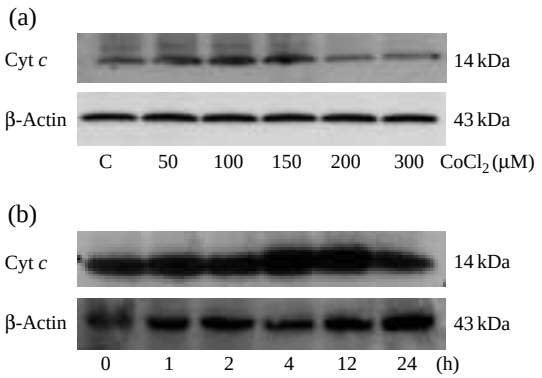

Fig. 5. Cytochrome *c* was released from mitochondria into cytoplasm in $CoCl_2$-treated PC12 cells. PC12 cells were incubated with indicated $CoCl_2$ concentrations (a) and with 150 μM $CoCl_2$ for indicated time (b). Cytoplasmic cytochrome *c* was analyzed by Western blot analysis. The levels of cytochrome *c* were quantitated densitometrically. Data is representative the mean ± SD for three independent experiments.

value at 12 h of incubation and returned to control values at 24 h (Fig. 5B). The results implicate that cytochrome *c* was released from mitochondria into cytoplasm during $CoCl_2$-induced apoptosis.

4. Caspases are involved in the $CoCl_2$-induced apoptosis in PC12 cells

Since it is important to identify the intracellular apoptotic pathways induced by $CoCl_2$ in PC12 cells, caspase activities were measured on the basis that active caspases consequently cleave their substrate at a specific site. Caspase-8 and caspase-9 activities were elevated in $CoCl_2$-treated PC12 cell. After exposure to $CoCl_2$, caspases activities were increased about 3 folds in caspase-8 and about 1.5 folds in caspase-9, compared with that of control respectively. Activation of caspase-9 was occured lately at 24 h with $CoCl_2$ incubation, compared with early activation of caspase-8 at 12 h (Fig. 6). $CoCl_2$-treated PC12 cells showed a significant increase in caspase-3 activity in time- and dose-dependent manners (Fig. 7).

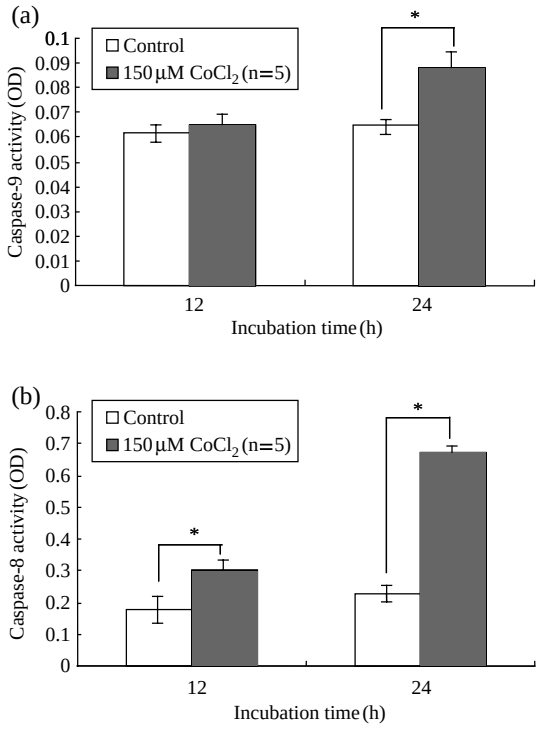

Fig. 6. Caspase-8 and -9 protease were activated by $CoCl_2$ in PC12 cells. Cells were treated with 150 μM $CoCl_2$ for 8 h and 24 h. Absorbance for caspase-9 activity was measured at 405 nm after incubation with LEHD-*p*NA substrate (200 μM) for 24 h at 37℃ (a). After incubation with reaction buffer containing IETD-AFC substrate for 1 h at 37℃, caspase-8 activity was measured by fluorometer using UV excitation of 400 nm and having an emission wavelength range of 480 ~ 520 nm (b). *$p < 0.05$ versus the corresponding control value.

5. Fas/APO-1 expression is increased in $CoCl_2$-treated PC12 cells

To clarify whether death recepter-mediated apoptosis pathway is activated in PC12 cells, the expression of Fas/APO-1, a death receptor, was observed using Western blot analysis. Fas/APO-1 expression was increased in $CoCl_2$-treated cells at 12 or 24 h of incubation, compared to that of untreated cells (Fig. 8). In addition, Bid expression in $CoCl_2$-induced apoptosis was determined since Bid activation is known to

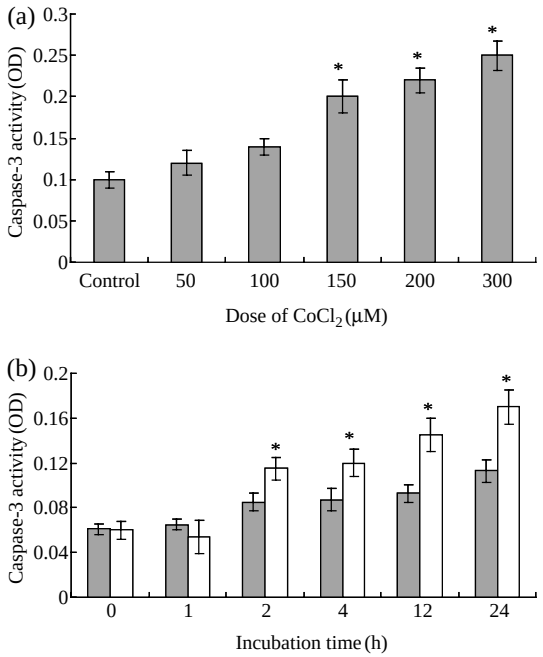

Fig. 7. Caspase-3 protease was activated by $CoCl_2$ in PC12 cells. After cells were treated with a different doses for 24 h (a) and for indicated times at 150 μM $CoCl_2$ (b), absorbance for caspase-3 activity was measured at 405 nm after incubation with DEVD-*p*NA substrate (200 μM) for 24 h at 37℃. $*p < 0.05$ versus the corresponding control value.

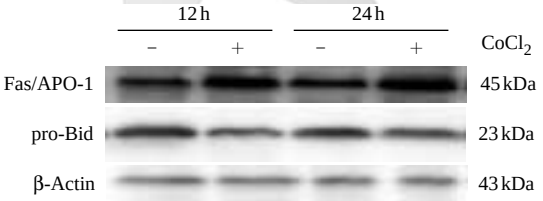

Fig. 8. Fas/APO-1 expression was upregulated and Bid-proform was decreased in $CoCl_2$-treated PC12 cells. After cells were treated with 150 μM for 12 h or 24 h, the protein levels of Fas/APO-1 and Bid were determined by Western blot analysis.

be at downstream of activated caspase-8. After 12 or 24 h incubation with $CoCl_2$, Western blot analysis using anti-Bid binding with full length Bid (pro-Bid) was performed. The levels of pro-Bid were decreased in $CoCl_2$-treated cells, compared with that of untreated cells (Fig. 8).

Discussion

Hypoxia/ischemia-induced cell death has been classified as apoptosis and necrosis, on the basis of changes in morphology, enzymatic activity, ATP concentration and adjacent cellular effects (Fowthrop et al. 1991, Levin 1998, Wang et al. 2001). The characteristic morphology in apoptotic cell is distinct, including cellular shrinkage, internucleosomal DNA fragmentation and chromatin condensation (Oppenheim 1991, Fujimura et al. 2000).

Previous study have shown that cobalt chloride induces apoptosis in PC12 cells through the production of ROS and accompanied by AP-1 activation (Strasser et al. 2000, Zou et al. 2001). In the present study, $CoCl_2$ induced the production of ROS in PC12 cells, and NAC, a free radical scavenger, abrogated ROS production. Besides, $CoCl_2$-treated cells demonstrated not only DNA fragmentation showing ladder pattern, but also morphologic changes such as cell swelling, condensed nuclei, and fragmented apoptotic nuclei. Taken together, it is suggested that $CoCl_2$ induce apoptosis of PC12 cells through ROS-mediated process. However, the intracellular apoptotic mechanisms have not been examined in $CoCl_2$-induced apoptosis.

One important question is which intracellular apoptotic regulators are involved in $CoCl_2$-induced apoptosis. Caspase-3 is a key and a common protease in both mitochondria- and death receptor-dependent pathways, and particularly important in neurons (Kuida et al. 1996, Earnshaw et al. 1999). Previous studies have shown that caspase-3 is activated and expressed in response to various hypoxia in PC12 cells, indicating that caspase-3 may play a pivotal role in hypoxia-induced apoptosis in PC12 cells (Yoshimura et al.

1998, 1999). Indeed, a recent study reported that caspase-3 like proteases are activated during the apoptotic cell death in $CoCl_2$-treated PC12 cells (Zou et al. 2002). The present study showed that caspase-3 activity was upregulated, which is consistent with that of the previous report. However, the upstream mechanism which activates caspase-3 has not been still examined in $CoCl_2$-induced apoptosis, even if some pathways are suggested in various hypoxic models.

Mitochondria has been known to serve as a main target in various hypoxic/ ischemic models (Li et al. 1997, Fujimura et al. 1998, Shen et al. 2001). One possible mechanism for activating caspase-3 in $CoCl_2$-induced apoptosis is caspase-9 mediated process activated by cytochrome *c* released from the mitochondria, in concert with Apaf-1 and dATP. Although mitochondrial cytochrome *c* release and caspase-9 activation have been reported in focal and global ischemia models (Fujimura et al. 1998, Araya et al, 1998), there are no reports to change in mitochondria function in $CoCl_2$-induced apoptosis. The present study presents the first evidence that mitochondria plays a pivotal role in $CoCl_2$-induced apoptosis. Caspase-9 activity was upregulated and cytochrome *c* was released from mitochondria into cytosol in $CoCl_2$-treated cells, suggesting that caspase-3 activation is in part mediated by mitochondria-dependent pathway in $CoCl_2$-induced apoptosis. There was an interesting result that cytochrome *c* released from mitochondria in low doses at below 150 μM $CoCl_2$, but was not released in high doses at over 150 μM $CoCl_2$. Thus, it is speculated that $CoCl_2$-induced cell death is driven from necrosis at high concentration. These results support that there is a strong causal link between mitochondrial dysfunction and caspase activation in $CoCl_2$-induced apoptosis. Recently, some reports have shown that a mitochondrial complex comprising the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocate (ANT) in the inner membrane and cyclophilin-D (Cyp-D) in the matrix, assembles at contact sites between the inner and outer membrane, subsequently forming the permeability transition (PT) pore, which can be open transiently, allowing free permeation of cytochrome *c* from the mitochondrial intermembrane space to the cytosol (Beutner et al. 1998, Crompton et al. 2000). Besides, recent reports have demonstrated that ROS leads to an induction of PT pore opening and a loss of $\Delta\psi_m$, thereby followed by release of cytochrome *c* into cytosol from mitochondria (Ankarcrona et al. 1995, Krajewski et al. 1999, Xia et al. 1999). From the previous and present studies, it is assumed that ROS produced by $CoCl_2$ impaires mitochondrial function accompanied by cytochrome *c* release, subsequently activating caspase-9.

The other possible mechanism for activating caspase-3 is caspase-8 mediated process activated by Fas/APO-1 and TNF receptor-1. Recent studies have reported that ROS such as H_2O_2 directly induces upregulation of Fas and Fasligand, subsequently activating caspase-8 (Fleury et al. 2002, Facchinetti et al. 2002). In addition, it was recently known that caspase-8 is critical to focal cerebral ischemia which induces apoptosis (Yin et al. 2002). From these previous reports, a possibility was proposed that death receptor-dependent apoptosis pathway may be involved in caspase-3 activation in $CoCl_2$-induced apoptosis (Zou et al. 2002). However, there are not established on the extrinsic pathway in $CoCl_2$-induced apoptosis. In the present study, Fas/APO-1, a death receptor, was upregulated and caspase-8 activity was enhanced in $CoCl_2$-treated cells. Taken together, $CoCl_2$-induced apoptosis is likely to be mediated by both mitochondria and death receptor-mediated pathways.

Another question remains as to which Bcl-2 family involved in $CoCl_2$-induced apoptosis of PC12 cells. In general, proteins of the Bcl-2 family are well-characterized regulators of apoptosis, consisting of three distinct subfamilies. The Bcl-2 subfamily contains antiapoptotic proteins such as Bcl-2 and Bcl-XL,

which reduce cytochrome *c* release (Gottlieb et al. 2000, Howard et al. 2002). The Bax subfamily contains proapoptotic proteins such as Bax and Bak, which induce cytochrome *c* release (Starkov et al. 2002). Bcl-2 proteins such as Bid, Bik and Bim are another subfamily of proapoptotic proteins, which are activated by caspase-8. Furthermore, ratio of proapoptotic and antiapoptotic Bcl-2 proteins may be pivotal cue to release of cytochrome *c* from mitochondria. Therefore, expression of Bcl-2 family was examined during $CoCl_2$-induced apoptosis to elucidate the involvement of Bcl-2 family in $CoCl_2$-induced apoptosis. In the present study, Bcl-2 mRNA was downregulated, whereas Bax mRNA was upregulated in $CoCl_2$-treated cells. These findings suggest that Bcl-2 proteins are involved in $CoCl_2$-induced apoptosis. An interesting result is that Bid was overexpressed by $CoCl_2$, since Bid is known to be activated by caspase-8, unlike other Bcl-2 family. Besides, activation of caspase-8 preceeded the activation of caspase-9 by $CoCl_2$. From these results, it was speculated that the death receptor-mediated apoptotic signals may regulate the mitochondria-mediated apoptotic signals. However, roles of Bcl-2 family may be debate in $CoCl_2$-induced apoptosis of PC12 cells since Bcl-2 family regulates the production of ROS and cytochrome *c* release from mitochondria in hypoxic/ischemic condition (Gottlieb et al. 2000, Starkov et al. 2002) and ROS could conversely regulates the expression of Bcl-X_L mRNA (Herrera et al. 2001). Further researches for the roles of the Bcl-2 family showed be needed in $CoCl_2$-induced neuronal apoptosis.

In summary, the present results suggest that $CoCl_2$ induces apoptosis through activation of both the mitochondrial- and death receptor pathway mediated by Bcl-2 family and caspases (-8, -9 and -3) in PC12 cells. Furthermore, the results of the present study will provide a molecular basis for understanding of physiological and pathological processes of the neuronal apoptosis in hypoxic/ischemic condition.

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Abstract

PC12 세포에서 $CoCl_2$ 유발 세포자멸에 대한 Bcl-2 family와 caspase의 역할

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간추림 : 허혈성/저산소 상태에서 신경손상을 유발하는 세포고사가 발생되는데, 이러한 신경세포자멸 기전 및 치료방법이 아직 정립되어 있지 않다. $CoCl_2$는 활성산소종(ROS)을 생산하는 등, 저산소 환경과 유사한 조건을 초래하는 것으로 알려져 있다. 본 연구의 목적은 PC12 세포에서 $CoCl_2$에 의한 저산소 상태에서 세포자멸기전에 대한 Bcl-2와 caspase들의 역할을 구명하는데 있다.

세포생장력은 MTT 방법으로 측정하였고, DNA 절편화 및 세포자멸 소체는 DNA laddering과 propidium iodide (PI) 염색법으로 조사하였다. Bcl-2와 Bax 발현정도는 RT-PCR법, Bid, Fas/APO-1 발현과 미토콘드리아에서 세포질로 분비된 cytochrome *c*는 Western blot으로 분석하였으며, caspase-3와 caspase-9 활성은 spectrophotometer 그리고 caspase-8의 활성은 fluorospectrometer에 의해 측정하였다.

$CoCl_2$ 투여는 PC12 세포수를 시간과 농도 의존적으로 감소시켰고, DNA 절편화 현상과 세포자멸 소체를 유도하였다. 또한, 미토콘드리아에서 세포질로 유리되는 cytochrome *c*양이 증가되었고, caspase-9와 -3의 활성이 증가되었다. 이러한 결과는 $CoCl_2$ 투여한 세포에서 유도된 세포자멸기전에 미토콘드리아가 매개되었음을 보여주었다. 한편, $CoCl_2$ 투여에 의해 Fas/APO-1의 발현이 증가되었고, caspase-8 활성이 증가된 결과로 $CoCl_2$ 유도된 세포자멸 기전에 death receptor 매개 신호경로와 연관되었음을 알 수 있었다. 또한, Bcl-2 family에 대한 RT-PCR 분석결과, 세포자멸를 억제하는 Bcl-2 발현은 감소되었으나, 세포자멸을 자극하는 Bax발현은 증가되었으며, 한편 pro-Bid 발현은 감소되었다.

따라서, 본 실험의 결과들은 PC12 세포에서 Bcl-2 family와 caspase들이 미토콘드리아와 death receptor를 매개하는 두 신호경로를 통해서 $CoCl_2$ 유도 세포자멸에 중요한 역할을 함을 시사하였다.

찾아보기 낱말 : PC12 세포, $CoCl_2$, 세포자멸, Caspase, Bcl-2 family, 미토콘드리아

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