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Inhibition of Hypoxia-induced Apoptosis in PC12 Cells by Estradiol

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Neuronal apoptotic events, which result in cell death, are occurred in hypoxic/ischemic conditions. Estradiol is a female sex hormone with steroid structure known to provide neuroprotection through multiple mechanisms in the central nervous system. This study was aimed to investigate the signal transduction pathway of $CoCl_2$-induced neuronal cell death and the inhibitory effects of estradiol. Administration of $CoCl_2$ decreased cell viability in both a dose- and time-dependent manner in PC12 cells. $CoCl_2$-induced cell death produced genomic DNA fragmentation and morphologic changes such as cell shrinkage and condensed nuclei. It was found that $CoCl_2$-treated cells increased the reactive oxygen species (ROS) as well as caspase-8, -9 and -3 activities. However, pretreatment with estradiol before exposure to $CoCl_2$ prevented the reduction in cell viability reduction and attenuated DNA fragmentation and morphologic changes caused by $CoCl_2$. Furthermore, the $CoCl_2$-induced increases of ROS levels and caspases activities were attenuated by estradiol. Gene expression analysis revealed that estradiol blocked the underexpression of the Bcl-2 and ameliorated the increase in the release of cytochrome *c* from mitochondria into cytoplasm and Fas-ligand (Fas-L) upregulated by $CoCl_2$. These results suggest that $CoCl_2$ induce apoptosis in PC12 cells through both mitochondria- and death receptor-mediated cell death pathway. Estradiol was found to have a neuroprotective effect against $CoCl_2$-induced apoptosis through the inhibition of ROS production and by modulating apoptotic effectors associated with the mitochondria- and death-dependent pathway in PC12 cells.

Key Words: Estradiol, Apoptosis, $CoCl_2$, Caspase, Bcl-2 family, Mitochondria

INTRODUCTION

Apoptosis in neuronal cells and tissues occurrs in hypoxic/ischemic conditions, consequently resulting in neuronal damage (Fujimura et al, 1998; Wang et al, 2000). Hypoxic/ischemic conditions have long been recognized as important mediators or modulators of apoptosis because these conditions resulted in 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). However, the exact apoptotic mechanisms triggered by hypoxic/ischemic conditions and their respective treatments are not thoroughly understood.

In general, apoptosis is driven from the activation of a family of cysteine proteases called caspases, which cleave a critical set of cellular proteins to initiate apoptotic cell death (Crompton, 2000; Roth et al, 2000). Proteins from these family are expressed as proenzymes and activated by upstream stimuli. Among at least 14 known mammalian caspases, 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). The two main pathways for activating caspases are classified as extrinsic pathway (death receptor-mediated mechanism) and intrinsic pathway (mitochondria-mediated mechanism) (Qin et al, 2001). Both pathways share the activation of caspase-3 as an executioner caspase, which activates caspase -activated DNase, causing apoptotic DNA fragmentation. The death receptor pathway is stimulated by a cell surface death receptor such as Fas or tumor necrosis factor (TNF) receptor. The receptors activated by ligands lead to activation of caspase-8, with subsequent activation of caspase-3. The mitochondrial pathway is stimulated by hypoxic/ischemic conditions, cytotoxic reagents, radiation, and growth factor deprivation (Shimizu et al, 1996). These stimuli induce generation of ROS, leading to the loss in mitochondrial transmembrane potential ($\Delta\Psi_{mm}$), and release of cytochrome *c* from mitochondria to the cytosol. The subsequent interaction of cytochrome *c* with Apaf-1 protein results in the recruitment of procaspase-9. Activation of caspase-9 leads to the activation of caspase-3 and subsequently contributes to apoptotic cell death. Besides caspase, members of the Bcl-2 family are also critical for the regulation

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ABBREVIATIONS: $CoCl_2$, cobalt chloride; PC12 cell, rat pheochromocytoma; ROS, reactive oxide species; NAC (N-acetyl-L-cystein); RT-PCR, Reverse Transcription Polymerase Chain Reaction, Fas-L; Fas-ligand; DCF-DA, 2', 7'- dichlorofluorescin diacetate.

of apoptosis (Tsujimoto & Shimizu, 2000). The Bcl-2 family controls the release of mitochondrial cytochrome *c* to the cytosol by modulating the permeability of the outer mitochondrial membrane. However, the roles of the Bcl-2 family and caspases in hypoxic/ischemic-induced neuronal apoptosis have not been well elucidated.

On the other hand, estradiol, a female sex hormone with steroid structure, is known to be important for the differentiation of specific hypothalamic nuclei and normal brain function. Studies in various systems have shown that estrogen protects neurons from adverse conditions or factors including beta-amyloid peptide (Green et al, 1996), serum-deprivation (Gollpudi & Oblinger et al, 1999), excitotoxicity (Singer et al, 1998), and oxidative stress (Moosmann & Behl, 1999). Furthermore, estrogen has been shown to provide neuroprotection through multiple mechanisms (Green et al, 2000; Garcia-Segura et al, 2001). However, estrogen-induced antiapoptotic mechanism in hypoxic/ischemic conditions remains unsettled. The main mammalian estrogen, 17β-estradiol (estradiol), is a major potent structure responsible for biological effects and is the most widely studied (Behl et al, 1997; Green et al, 1997). Thus, estradiol is expected to have beneficial effects under neuronal hypoxic conditions that trigger the production of ROS, and subsequently result in neuronal apoptosis.

There are some reports showing that cobalt chloride ($CoCl_2$) could mimic hypoxic/ischemic conditions, including generation of reactive oxygen species (ROS) and transcriptional change of some genes such as hypoxia inducible factor (HIF-1), p53, p21 and pCNA (Chandel et al, 1998; Chandel & Schumacker, 1999; Guichun et al, 2000; Wang et al, 2000). PC12 is a cell line derived from rat pheochromocytoma and is widely used as an *in vitro* model for investigating neuronal apoptosis and neuronal differentiation (Kroll & Czyzyk-Krzeska, 1998).

On the basis that $CoCl_2$ mimics the hypoxic/ischemic condition, this study was designed to investigate the antiapoptotic effects of estradiol on $CoCl_2$-induced apoptosis in PC12 cells.

METHODS

Cell culture and $CoCl_2$ and estradiol treatment

The rat pheochromocytoma PC12 cells, were maintained in RPMI 1640 medium containing 10% horse serum, 5% heat-inactivated fetal bovine serum (Gibco BRL, USA) and gentamicin (50 μg/ml, Gibco BRL, USA) under 5% CO_2 at 37°C. Estradiol (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma, USA). Cells were pretreated with estradiol or NAC (N-acetyl-cysteine) for 1 h prior to $CoCl_2$ treatment.

Cell viability assay by MTT assay

The MTT assay relies on the observation that viable cells with active mitochondria reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a visible dark-blue formazan reaction product which provides an indirect measurement of cell viability. PC12 cells were plated onto 96 well plates and exposed to $CoCl_2$ alone or pretreated with different concentrations of estradiol for 1 h. After estradiol and $CoCl_2$ treatments, MTT was added to the culture medium in respective times at a final concentration of 0.1 mg/ml and incubated at 37°C for 4 h. The reaction product of MTT was extracted in DMSO and optical density (OD) was spectrophotometrically measured at 570 nm using an ELISA reader (ELx800uv; Bio Tek Instruments, USA).

Nuclear staining with Diff-Quick

Morphological changes of apoptotic cells were investigated by Diff-Quick stain. Cells plated in 60 mm dishes at a density of 1×10^6 were treated with 150 μM $CoCl_2$ or estradiol for 1 h before $CoCl_2$ treatment if necessary. The treatments were continued for 24 h, and then the cells were washed with PBS and fixed with acetone and methanol (1 : 1). After incubating for 20 min at −20°C, cells were stained with Diff-Quick (Kuk Jae, Japan) solution and observed under microscope (Olympus, Japan).

Agarose gel electrophoresis for DNA fragmentation

Oligonucleosomal fragmentation of genomic DNA was assessed using the Apopladder kit (TaKaRa Shuzo, Japan) according to the manufacturer's instructions. Cells were plated in 60 mm dishes at a density of 1×10^6 and then preincubated for 1 h with or without estradiol, followed by incubation with 150 μM $CoCl_2$. Briefly, cells were lysed in 200 μl of lysis buffer and centrifuged at 1,100 g for 10 min. The supernatant was then incubated at 56°C for 1 h followed by the addition of 20 μl of 10% SDS and 20 μl of proteinase K (20 mg/ml), and then treated with 1 μg of RNase A at 37°C for 1 h. DNA was extracted and precipitated overnight at −20°C in a precipitant mixture containing 0.95 ml ethanol and pelleted by centrifugation for 15 min at 12,000 g at 4°C. 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. DNA bands were visualized under UV light after staining with ethidium bromide.

Detection of ROS production

ROS production was monitered with a fluoresence spectrometer (Hitachi F-4500, japan) using 2', 7'- dichlorofluorescin diacetate (DCF-DA). Cells were plated on 96-well plate and treated with NAC. DCF-DA (25 μM) was added into the medium and incubated for 10 min at 37°C. Exitation was measured at 485 nm and emission was measured at 530 nm.

Measurement of caspase assay

Caspase activity was assayed using the caspase-3, -9 activity assay kit (Calbiochem, CA) and caspase-8 activity kit (BD, USA) according to the manufacturer's instructions. Briefly, PC12 cells were grown on 100 mm dishes and treated with $CoCl_2$ alone or pretreated with estradiol for the indicated time. The media were removed from the culture dishes and the cells were collected and washed with PBS, and then resuspended in cell lysis buffer. After incubation on ice for 10 min, the lysates were centrifuged for 20 min at 10,000 g, and the supernatants were collected and protein concentrations were determined (BCA assay, Pierce). Fifty microliters of cell lysates were mixed with reaction buffer containing the DEVD-*p*NA substrate (200 μM) for caspase-3 activity, LEHD-*p*NA substrate (200 μM)

for caspase-9 activity and IETD-*p*NA substrate for caspase-8 activity. After incubation for 24 h at 37°C, each absorbance in each well measured at 405 nm with an ELISA reader.

Isolation of total RNA and Reverse transcription polymerase chain reaction (RT-PCR)

For extraction of total RNA, cells were homogenized with a polytron homogenizer in Trizol reagent (Gibco-BRL, USA) according to the manufacturer's instructions. RNA samples were quantified by spectrophotometry at 260 nm wavelength. For synthesis of cDNA, 2 μg of total RNA and 2 μl of Oligo (dT) (10 pmole) were mixed with 50 μl RNase-free water, and then incubated at 42°C for 1 h and 94°C for 5 min. PCR products were generated in PCR buffer containing 10 pmole of each primer by using a PCR-premix kit (Bioneer, Korea). After the first denaturation step (5 min at 95°C), samples were subjected to 30 cycles consisting of 40 sec at 95°C, 40 sec at 55°C, and 1 min 30 sec at 72°C, with a final extention step of 10 min, on a GeneAmp PCR system (Perkin-Elmer 2400, USA). The following primer pairs were used: for Bax, 5'-GTTTCATCCAGGATCGAGC-AG-3' (sense primer) and 5'-CATCTTCTTCCAGATGGTG-A-3' (antisense primer); for Bcl-2, 5'-CCTGTGGATGACTG-AGTACC-3' (sense primer) and 5'-GAGACAGCCAGGAGA-AATCA-3' (antisense primer); for Fas-L, 5'-CAGCCCCTG-AATTACCCATG-3' (sense primer) and 5'-CACTCCAGAG-ATCAAAGCAG-3' (antisense primer). The amplified products were analyzed on a 1.5% agarose gels containing ethidium bromide and visualized by UVP Transilluminator /Polaroid camera System (UVP Laboratories, CA). 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 band intensities were determined using the NIH Scion Image Software.

Western blotting

Cells were washed twice with PBS and proteins were solubilized in lysis buffer (500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzamidin, 1 μg/ml Trypsin inhibitor) containing a cocktail of protease inhibitor (Roch, Germany). Lysates were centrifuged at 10,000×g and protein concentrations were determined by BCA protein assay (Pierce, IL). Proteins were electroblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and incubated with the respective primary antibody. Rat monoclonal antibodies against cytochrome *c* (Pharmingen, CA) were applied. Blots were incubated with specific peroxidase-coupled secondary antibodies (anti-mouse IgG horseradish peroxidase [HRP], anti-Rabbit IgG-HRP, Sigma, USA). Bound antibodies were visualized using an enhanced chemiluminescent detection system (Amersham Pharmacia Biotech, UK).

Statistical analysis

Results are expressed as mean±standard error (S.E.) and analyzed by student's *t*-test. Mean values were considered significantly different at $p<0.05$.

RESULTS

EGCG attenuates the $CoCl_2$-induced decreased in cell viability

Treatment with $CoCl_2$ resulted in a concentration and a time -dependent decrease of cell viability in PC12 cells (Fig. 1A~B). The survival rate of PC12 cells was about 50% when cells were treated with 150 μM $CoCl_2$ for 8 h. Pre-incubation of PC12 cells with 1-500 nM of estradiol for 1 h inhibited $CoCl_2$-induced cell death. The effective dose of estradiol for rescuing cell viability at approximately 85 % was 100 nM, among the explored concentrations of EGCG in $CoCl_2$-treated PC12 cells (Fig. 1C).

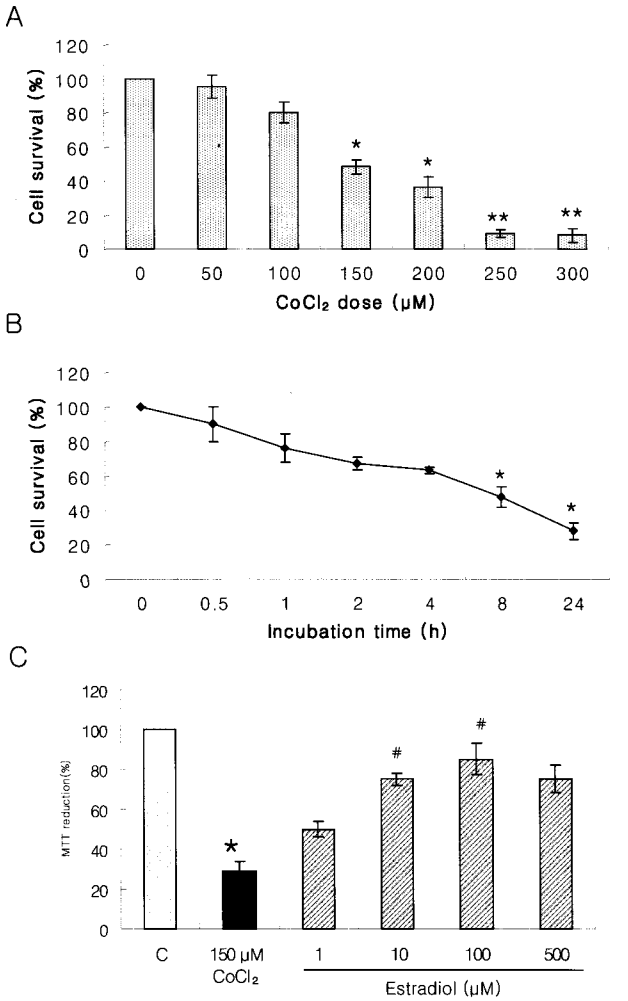

Fig. 1. Effects of estradiol and $CoCl_2$ on the viability in PC12 cells. PC12 cells were incubated in medium containing different concentrations of $CoCl_2$ for 8 h (A), or incubated for different periods at 150 μM $CoCl_2$ (B), or with pretreatment of estradiol for 1 h prior to 150 μM $CoCl_2$ treatment for 8 h (C). Cell viability was estimated by MTT assay. Each value represents the mean ±S.E. from 5 independent experiments. $^*p<0.05$, $^{**}p<0.01$ relative to control, $^{\#}p<0.01$ relative $CoCl_2$.

Estradiol suppresses $CoCl_2$-induced apoptosis

Morphologic changes associated with apoptosis were observed using a Diff-Quick staining after 150 μM $CoCl_2$ exposure for 24 h (Fig. 2A). Cells exposed to $CoCl_2$ revealed cell shrinkage, blebbing of plasma membrane, chromatin condensation and apoptotic nuclei. However, pretreatment with 100 nM estradiol for 1 h before $CoCl_2$ exposure reduced the number of cells with apoptotic morphological changes. To confirm whether $CoCl_2$ induced the apoptosis in PC12 cells, genomic DNA was analyzed by agarose gel electrophoresis. Treatment with 150 μM $CoCl_2$ for 24 h resulted in the formation of oligonucleosome-sized fragments of DNA displayed as a ladder, whereas control cells without $CoCl_2$ treatment did not show DNA laddering. Pretreatment with estradiol attenuated $CoCl_2$-induced DNA fragmentation in PC12 cells (Fig. 2B).

Estradiol prevents $CoCl_2$-induced oxidative stress

The degree of ROS generation by $CoCl_2$ was measured by fluorescence assay using DCF-DA probe. DCF-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals, mainly H_2O_2, and convert into

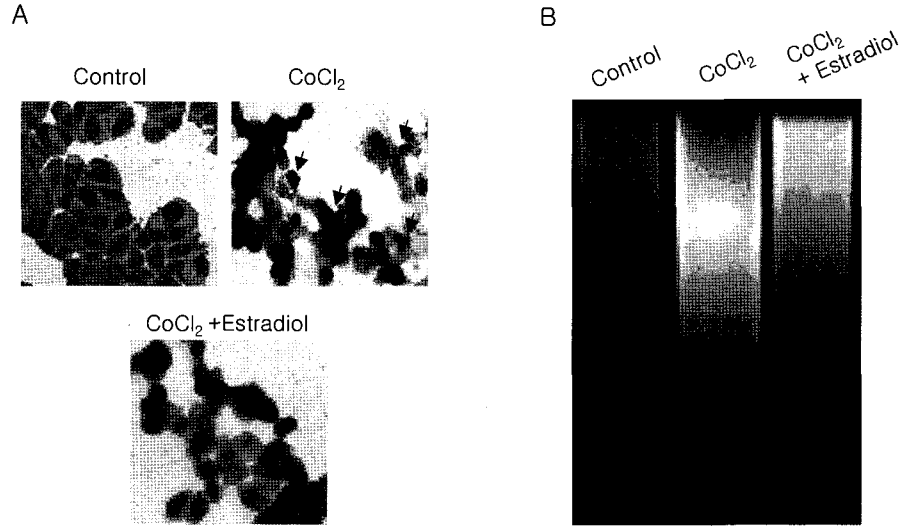

Fig. 2. Effect of estradiol on $CoCl_2$-induced apoptosis in PC12 cells. PC12 cells were incubated with or without $CoCl_2$ (150 μM, 8 h), or estradiol pretreatment (100 nM, 1 h). The cells were stained with Diff-Quick method and nuclear morphology was detected by microscopy (×400). Arrows indicate the typical apoptotic cell (A). DNA was prepared from the cells treated with 150 μM $CoCl_2$ for 24 h or pretreated with 100 nM estradiol for 1 h and genomic DNA was electrophoresed and visualized with ethidium bromide (B).

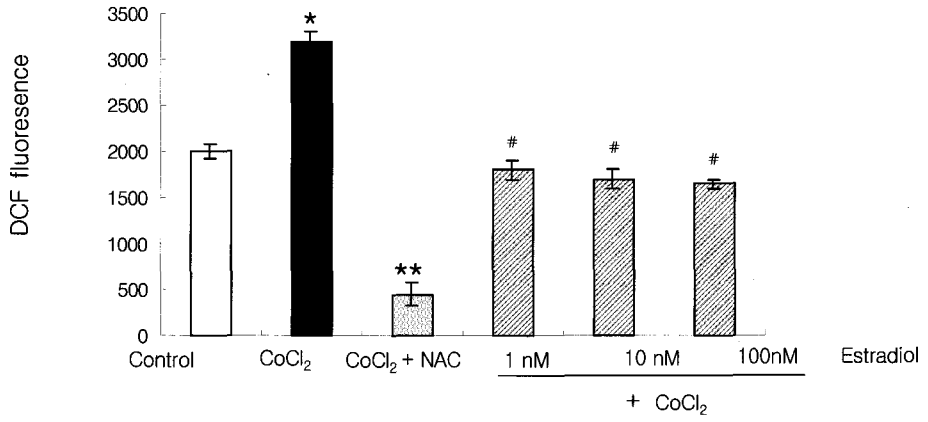

Fig. 3. Effects of estradiol on ROS production in $CoCl_2$-treated PC12 cells. PC12 cells loaded with DCF-DA were incubated with $CoCl_2$ alone or co-incubation with 5 mM NAC or estradiol for 1 h. The intracellular levels of ROS were detected by measuring the DCF fluorescence. Each value represents the mean±S.E. from 5 independent experiments. $^*p<0.05$, $^{**}p<0.01$ relative to control, $^{\#}p<0.01$ relative to $CoCl_2$.

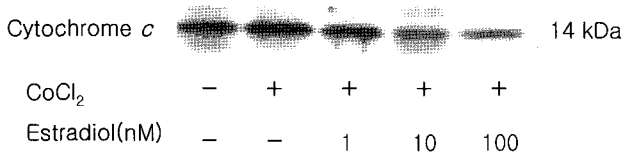

Fig. 4. Effect of estradiol on the expression of Bcl-2 and Bax in $CoCl_2$-treated PC12 cells. The mRNA levels of Bax and Bcl-2 were determined by RT-PCR analysis in $CoCl_2$ (150 μM)-treated PC12 cells for 8 h with or without pretreatment of estradiol for 1 h. The ratio of Bax to Bcl-2 was determined by densitometry and plotted. Each value represents the mean ±S.E. from 5 independent experiments.

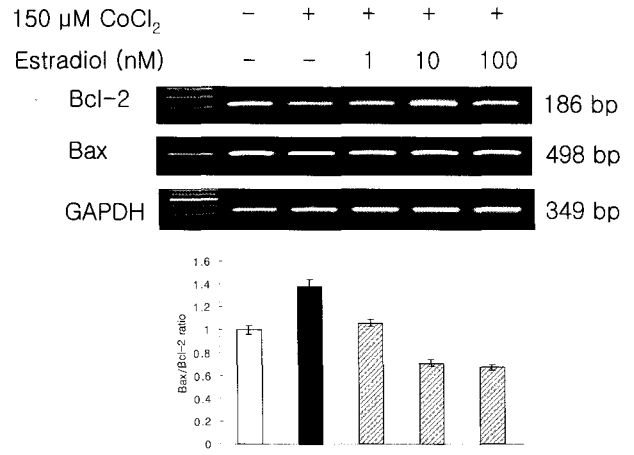

Fig. 5. Inhibition of $CoCl_2$-induced cytochrome *c* release by estradiol. Levels of cytosolic cytochrome *c* (14 kDa) were determined using western blot in PC12 cells exposed to $CoCl_2$ (150 μM, 8 h) with or without pretreatment of estradiol for 1 h.

its fluorescent products, which are retained within the cell. Thus, this method provides an index of cell cytosolic oxidation. As shown in Fig. 3, treatment with 150 μM $CoCl_2$ for 8 h enhanced the production of ROS in PC12 cells. Pretreatment with estradiol (1, 10, and 100 nM) for 1 h significantly attenuated $CoCl_2$-induced intracellular ROS to a lower level than control group, and similar to NAC (5 mM), a ROS scavenger.

Estradiol ameliorates the Bcl-2 downregulation triggered by $CoCl_2$

The balance between gene expression of pro-apoptotic and anti-apoptotic member in Bcl-2 family is crucial in determining the fate of cells that undergo apoptosis. To investigate the effects of $CoCl_2$ and estradiol on the expression of Bcl-2 family, RT-PCR was performed in PC12 cells treated with 150 μM $CoCl_2$ or with an estradiol pretreatment prior to $CoCl_2$ exposure. Expression of Bcl-2, an inhibitor of cytochrome *c* release from mitochondria into cytoplasm, was downregulated by treatment with 150 μM $CoCl_2$. However, expression of Bax, an inducer of cytochrome *c* release, was not changed in $CoCl_2$-treated cells (Fig. 4). Estradiol pretreatment (1, 10, and 100 nM) for 1 h before exposure to $CoCl_2$ ameliorated the $CoCl_2$-induced downregulation of Bcl-2, but had no effect on Bax expression.

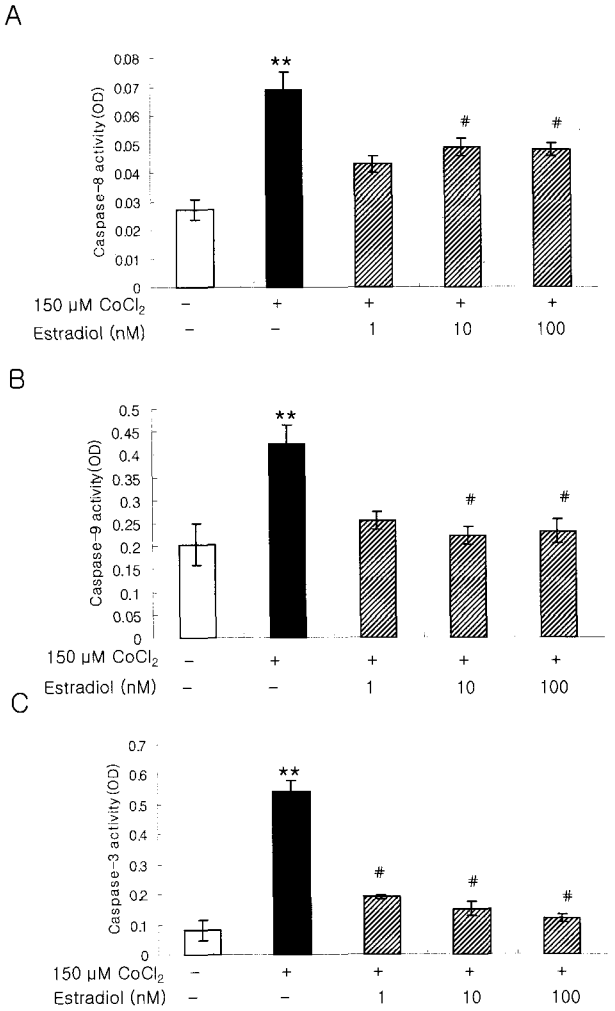

Fig. 6. Effect of estradiol on the activity of caspases in $CoCl_2$-treated PC12 cells. Enzymatic activity of caspase protease was determined by incubation of 50 μg total protein with IETD-*p*NA, LEHD-*p*NA, and DEVD-*p*NA substrates (200 μM) in $CoCl_2$ (150 μM, 8 h)-treated PC12 cells, without or with pretreatment of estradiol for 1 h. Each value represents the mean ±S.E. from 5 independent experiments. $^{**}p<0.01$ relative to control, $^{\#}p<0.01$ relative to $CoCl_2$.

Estradiol blocks the release of cytochrome c from mitochondria into cytosol

To evaluate whether mitochondrial dysfunction is involved in $CoCl_2$-induced apoptosis or not, release of cytochrome *c* from mitochondria into cytosol was detected. Cytosolic cytochrome *c* was upregulated when PC12 cells were exposed to 150 μM $CoCl_2$ for 8 h, indicating that cytochrome *c* was released from mitochondria into cytoplasm during $CoCl_2$-induced apoptosis. However, an estradiol treatment prior to additiion of $CoCl_2$ attenuated the $CoCl_2$-induced increase of cytochrome *c* release (Fig. 5).

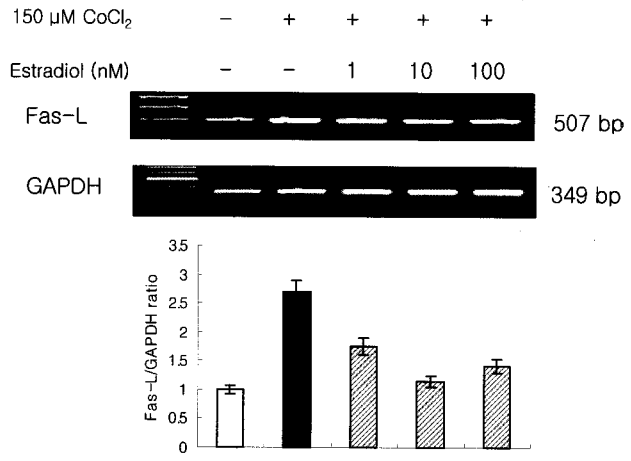

Fig. 7. Effect of estradiol on Fas and Fas-L expression in $CoCl_2$-treated PC12 cells. Cells were incubated with 150 μM $CoCl_2$ for 8 h with or without pretreatment of estradiol for 1 h. Each value represents the mean±S.E. from 5 independent experiments.

Estradiol inhibites the $CoCl_2$-induced increase in caspase activity

To determine whether caspases are involved in $CoCl_2$-induced apoptosis, the activities of caspase-8 and -9, which are initiating caspases, and caspase-3, an executing caspase, were measured. After treatment with 150 μM $CoCl_2$, the activities of caspase-8, -9, and -3 were enhanced by, 2.5, 2.1, and 5.5-folds, respectively, when compared with control groups. Addition of estradiol prior to $CoCl_2$ treatment was found to attenuate $CoCl_2$-induced caspase activities, to levels similar to that of the control (Fig. 6).

Estradiol modulates the $CoCl_2$-induced increased Fas expression

Fas-L, a death receptor-assemble, provides a link between death receptor-mediated signal and mitochondria-mediated pathway. To determine the involvement of a death-dependent apoptotic pathway in $CoCl_2$-induced apoptosis in PC12 cells, mRNA levels of Fas-L were determined by RT-PCR. As shown in Fig. 7, Fas-L was upregulated by $CoCl_2$ in PC12 cells. However, estradiol pretreatment prior to $CoCl_2$ exposure attenuated the $CoCl_2$-induced Fas-L upregulation by about 50%.

DISCUSSION

Hypoxia-induced cell death is a major concern in various clinical entities such as ischemic disease, organ transplantation, and other diseases. However, the mechanisms underlying the hypoxia-induced cell death and its treatment methods are still undefined. $CoCl_2$-induced apoptosis may serve as a simple and convenient *in vitro* model of hypoxia-induced apoptosis to elucidate molecular mechanism in hypoxia-linked cell death and search its treatment methods because $CoCl_2$ mimics hypoxic/ischemic condition including ROS production.

On the basis of changes in morphology, enzymatic activity, ATP concentration and adjacent cellular effects, hypoxia-induced cell death can be classified into two basic forms, apoptosis and necrosis, (Fowthrop et al, 1991; Levin, 1998). In the present study, $CoCl_2$ reduced PC12 cell viability in a concentration- and time-dependent manner. These results have also shown that $CoCl_2$ induced cell death like apoptosis showing the morphology features such as cell shrinkage, DNA fragmentation of ladder pattern, and chromatin condensation. Taken together, the main characteristics of $CoCl_2$ induced apoptosis in PC12 cells are in agreement with effects of hypoxia described in previous studies (Zou et al, 2001).

In the present study, $CoCl_2$ increased the intracellular ROS, while pretreatment with NAC, free radical scavenger, attenuated the increase of ROS level by $CoCl_2$. From these results, it is speculated that $CoCl_2$-induced apoptosis is at least partly driven from the production of ROS. However, the question pertaining how ROS is produced or regulated by $CoCl_2$ remains.

Recent reports have shown that the Bcl-2 family regulates the production of ROS and the release of cytochrome *c* from mitochondria into the cytosol under hypoxic/ischemic conditions (Gottlieb et al, 2000; Starkov et al, 2002). In general, the Bcl-2 family of proteins are well-characterized regulators of apoptosis, consisting of three distinct subfamilies. The Bcl-2 subfamily contains antiapoptotic proteins such as Bcl-2 and Bcl-X_L, which reduce cytochrome *c* release and a loss of $\Delta\Psi_m$ (Gottlieb et al, 2000; Howard et al, 2002). The Bax subfamily contains proapoptotic proteins such as Bax and Bak, which induce cytochrome *c* release and a loss of $\Delta\Psi_m$ (Starkov et al, 2002). The Bcl-2 family such as Bid, Bik and Bim are another subfamily of proapoptotic proteins, which are activated by caspase-8. In the present study, Bcl-2 mRNA was underexpressed, but Bax mRNA did not changed in $CoCl_2$-treated cells. These results indicated that $CoCl_2$ increased the Bax/Bcl-2 ratio, which favors apoptosis, while estradiol decreased the Bax/Bcl-2 ratio, subsequently resulting in the prevention of apoptosis. In addition, it is speculated that $CoCl_2$ downregulates Bcl-2, leading to the subsequent rise in ROS production, which in turn activates extrinsic and intrinsic apoptotic pathways. However, the roles of Bcl-2 family in the production of ROS may be in a debate, since ROS can conversely induce a decrease in Bcl-X_L mRNA (Herrera et al, 2001) and the release of cytochrome *c* can trigger accumulation of ROS (Cai & Jones, 1998).

Caspase-3 is a key executioner caspase involved in neuronal apoptosis. Previous studies have suggested that caspase-3 may pivotal role in hypoxia-induced apoptosis in PC12 cells, since it has been shown to be activated in response to various hypoxia and expressed in this cell type. Indeed, Zou *et al.* 2002) reported that caspase-3-like proteases are activated during the apoptotic cell death in $CoCl_2$-treated PC12 cells. The present results showed that caspase-3 activity was upregulated in $CoCl_2$-treated cells, which is consistent with the findings of Zou *et al.* (2002). These results suggest that $CoCl_2$-induced apoptosis is involved in a caspase-3 mediated mechanism. Caspase-3 activity is controlled by upstream regulators, such as caspase-8 or caspase-9, which modulates the mitochondria- and death receptor-dependent pathway, respectively and are important particularly in neurons (Kuida et al, 1996). However, the upstream mechanism that activates caspase-3 has not been still elucidated in $CoCl_2$-induced apoptosis, although some pathways are suggested in various models of hypoxia.

Because mitochondria are known to serve as the main target in various hypoxic/ischemic models, a possible mechanism for activating caspase-3 in $CoCl_2$-induced apoptosis could be related to the caspase-9 mediated process in response to the release of cytochrome *c* from the mitochondria, in concert with Apaf-1 and dATP because mitochondria have been known to serve as a main target in various hypoxic/ischemic model (Li et al, 1997; Fujimura et al, 1998; Shen et al, 2001). Although mitochondrial cytochrome *c* release into the cytosol and caspase-9 activation have been reported in focal and global models of ischemia (Fujimura et al, 1999), no report has referred to a change in mitochondrial function in $CoCl_2$-induced apoptosis of PC12 cells. In the present study, caspase-9 activity was upregulated and cytochrome *c* was released from mitochondria into the cytosol in $CoCl_2$-treated cells, suggesting that caspase-3 activation is in part mediated by a mitochondria-dependent pathway in $CoCl_2$-induced apoptosis. These results show that mitochondria plays a pivotal role in $CoCl_2$-induced apoptosis. Furthermore, recent reports have demonstrated that ROS leads to an induction of PT pore opening and a loss of $\Delta\Psi_m$ under ischemia and its related condition (Ankarcrona et al, 1995; Krajewski et al, 1999; Xia et al, 1999). From the previous and present studies, it is suggested that ROS produced by $CoCl_2$ impaires mitochondrial function, followed by a loss of $\Delta\Psi_m$ and release of cytochrome *c*, subsequently activating caspase-9.

It is shown that the pathway of death receptor-mediated apoptosis is related to caspase-8 activation. Once death receptors are activated by their ligation, they recruit the adaptor molecule, Fas associated death domain (FADD), which is followed by the activation of caspase-8. Recent studies have reported that ROS such as H_2O_2 directly induces upregulation of Fas and Fas-L, subsequently activating caspase-8 (Facchinetti et al, 2002; Fleury et al, 2002). Therefore, it has been speculated that ROS induced by $CoCl_2$ may regulate upregulation of Fas-L in PC12 cells. From these previous reports, it is suggested that the pathway leading to death receptor-mediated apoptosis may be involved in caspase-3 activation in $CoCl_2$-induced apoptosis. The present results demonstrated that Fas ligand levels were upregulated and caspase-8 activities were increased in $CoCl_2$-treated PC12 cells, showing the evidence that the death receptor-mediated pathway is involved in $CoCl_2$-induced apoptosis. From these findings mentioned above, $CoCl_2$-induced apoptosis is likely to be mediated by both intrinsic and extrinsic apoptosis pathways.

Estradiol is a classical sex steroid hormone, known to play an important and diverse roles in the neuronal system. It has been recently reported that estrogen plays a crucial role as a neurotrophic/neuroprotective agent (Moosmann, 1999; Green & Simpkins, 2000). Cell culture studies have shown that estrogen can improve neuronal viability and attenuates cell death induced by the neuropathology related to Alzeheimer's disease related neuropathology which is known to be driven from oxidative stress. Several reports have documented that estradiol attenuates β-amyloid-induced neurotoxicity in cultured hippocampal neurons and reduces 6-hydroxydopamine-induced apoptosis in PC12 cells (Chae et al, 2001; Hosoda et al, 2001). However, there are no previous reports refered to the neuroprotective effects of estradiol against $CoCl_2$-induced neuronal apoptosis. The present study is the first report documented that estradiol has a neuroprotective effect against $CoCl_2$-induced neuronal apoptosis, after remarkably preventing the loss in cell viability and inhibiting DNA fragmentation and morphologic changes related to apoptosis such as cell shrinkage and condensed nuclei in $CoCl_2$-treated PC12 cells. These present results suggest that estradiol has a neuroprotective effects by inhibiting the neuronal apoptosis in $CoCl_2$-induced neurotoxicity.

Estrogens have long been recognized as antioxidants in a variety of *in vitro* and *in vivo* models (Keller et al, 1997). The antioxidant property of estradiol is related to the phenolic A ring of the steroid structure (Behl et al, 1997; Green et al, 1997). In the present study, a low concentrations of estradiol (100 nM) attenuated accumulation of ROS production and prevented the cell death in $CoCl_2$-treated cells. Therefore, estrogen may function as an antioxidant through its direct or indirect scavenging of ROS in $CoCl_2$-induced apoptosis.

Members of the Bcl-2 family are important apoptotic regulators and therefore potential targets for the neuroprotective actions of estrogen (Singer et al, 1998; Dubal et al, 1999). Previous reports have shown that estrogen increases the expression of Bcl-2 in the MCF-7 breast cancer cell line, the NT-2 neuronal cell line, and cortical neurons (Huang et al, 1997; Singer et al, 1998). In addition, it was showed that estradiol may directly affect transcription of the Bcl-2 gene, since several putative estrogen-responsive sites are present in the Bcl-2 promoter (Teixeira et al, 1995). In the present study, RT-PCR analysis showed that estradiol increased the ratio of Bax to Bcl-2, by attenuating the $CoCl_2$ induced-downregulation of Bcl-2. These results suggest that estradiol inhibits $CoCl_2$- induced apoptosis through the regulation of the Bcl-2 family. Furthermore, estradiol attenuated the $CoCl_2$-induced increase in caspase-9 and caspase-3 activities as well as cytochrome *c* release. These results indicate that the neuroprotective effects of estradiol may result from the inactivation of caspase cascade associated with mitochondria pathway on $CoCl_2$-mediated apoptosis.

Finally, estradiol attenuated the increase in Fas-L expression, ligand binding with a death receptor, and the increase in caspase-8 activity by $CoCl_2$. This present study shows the evidence in which estradiol inhibits the death receptor-mediated apoptotic pathway through the downregulation of Fas-L, and the inhibition of caspase-8 activity. Further researches will be required to determine whether the neuroprotective effect of estradiol is mediated through the estrogen receptor.

In summary, pretreatment with estradiol attenuated apoptotic cell death, through antioxidant effect as well as modulating the release of cytochrome *c* from mitochondria into the cytosol, decreasing the Bax/Bcl-2 ratio, decreasing the expression of Fas-L, and inhibiting caspase-3, 8, and 9 activities induced by $CoCl_2$ in PC12 cells. The present results suggest that at physiological concentrations, estradiol is able to prevent hypoxia-induced apoptosis in neuronal cells by scavenging ROS and regulating effectors associated with the mitochondrial and death receptor-dependent pathways.

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