Effects of epigallocatechin gallate on $CoCl_2$-induced apoptosis in PC12 cells

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Abstract

Neuronal apoptotic events, consequently resulting in neuronal cell death, are occurred in hypoxic/ischemic condition. This cell death has been shown to be accompanied with the production of reactive oxygen species (ROS), which can attack cellular components such as nucleic acids, proteins and phospholipid. However, the underlying mechanisms of apoptosis induced in hypoxic/ischemic condition and its treatment methods are unsettled. Cobalt chloride ($CoCl_2$) has been known to mimic hypoxic condition including the production of ROS. Epigallocatechin gallate (EGCG), a green tea polyphenol, has diverse pharmacologial activities in cell growth and death. This study was aimed to investigate the apoptotic mechanism by $CoCl_2$ and effects of EGCG on $CoCl_2$-induced apoptosis in PC12 cells.

Administration of $CoCl_2$ decreased cell survival in dose- and time-dependent manners and induced genomic DNA fragmentation. Treatment with 100 μM EGCG for 30 min before PC12 cells were exposed to 150 μM $CoCl_2$, being resulted in the cell viability and DNA fragmentation being rescued. $CoCl_2$ caused morphologic changes such as cell swelling and condensed nuclei, whereas EGCG attenuated morphologic changes by $CoCl_2$. EGCG suppressed the apoptotic peak and a loss of $\Delta\psi_m$ induced by $CoCl_2$. $CoCl_2$ decreased Bcl-2 expression but Bax expression was not changed in $CoCl_2$- treated cells. EGCG attenuated the Bcl-2 underexpression by $CoCl_2$. $CoCl_2$ augumented the cytochrome c release from mitochondria into cytoplasm and increased caspase-8, -9 and caspase-3 activity, a marker of the apoptotic executing stage. EGCG ameliorated the incruement in caspase-8, -9 and -3 activity, and cytochrome c release by $CoCl_2$. NAC (N-acetyl-cysteine), a scavenger of ROS, attenuated $CoCl_2$-induced apoptosis in consistent with those of EGCG.

These results suggest that $CoCl_2$ induces apoptotic cell death through both mitochondria- and death receptor-dependent pathway and EGCG has neuroprotective effects against $CoCl_2$-induced apoptosis in PC12 cells.

Key words : $CoCl_2$, Apoptosis, EGCG, Mitochondria dependent pathway, Death receptor dependent pathway

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I. INTRODUCTION

Apoptosis is a gene-regulated mechanism of cell death that plays a pivotal role in physiological and pathological processes[1]. Apoptosis in neuronal cells and tissues, consequently resulting in neuronal dam-

age, is occurred in hypoxic/ischemic condition[2,3]. Hypoxic/ischemic condition has long been recognized as important mediators or modulators of apoptosis because this condition is accompanied with the production of reactive oxygen species (ROS) which can attack nucleic acids, proteins and membrane phospholipids[2,4,5].

In general, apoptosis is driven from the activation of a family of cysteine protease called caspases, which then cleave a critical set of cellular proteins to initiate apoptotic cell death[6,7]. Two categories of caspase responsible for apoptosis have been recognized: 1) the initiator caspase such as caspase-8, -9 and -10, which are activated in the earlier phase of apoptosis; 2) the executioner caspases such as caspase-3, -6, and -7, which are responsible for dismentling of cells[8]. At least two distinct pathways are involved in apoptosis. Both share activation of executioner caspase, caspase-3. Caspase-3 activates caspase -activated DNase which causes apoptotic DNA fragmentation. The intrinsic pathway (mitochondria-dependent pathway) is stimulated by hypoxic/ischemic condition, cytotoxic reagents, radiation, and growth factor deprivation[9,10]. These stimuli release cytochrome *c* from mitochondria into cytosol, subsequently resulting in caspase-9 activation which causes the activation of caspase-3. The exrinsic pathway (death receptor pathway or mitochondria-independent pathway) is stimulated by cell surface death receptors such as tumor necrosis factor (TNF) receptor and Fas. The receptors activated by ligands lead to caspase-8 activation, with subsequent activation of caspase-3. However, the exact apoptotic mechanisms in hypoxic/ischemic condition and its treatments are still unsettled.

On the other hand, tea polyphenols are natural plant flavonoids found in the leaves and stem of tea plant. The green tea polyphenols have showed a variety of pharmacological properties such as antiinflammatory, anticarcinogenic, and antioxidant effects[11-13]. The green tea polyphenols comprise (-)-epigallocatechin-3-gallate (EGCG), (-)- epigallocatechin, (-)-epicatechin, (+)-gallocatechin, and catechin. Among these polyphenols, EGCG is a major strong component responsible for biological effects. EGCG has been reported to display a potent antioxidant property because it possesses two triphenolic groups in its structure[14]. Thus, EGCG is expected to have beneficial effects in neuronal hypoxic condition accompanied with the production of ROS, subsequently resulting in neuronal apoptosis.

There are some reports showing that cobalt chloride ($CoCl_2$) could mimic the hypoxic/ischemic condition including the production of ROS in various cultured cells[15,16]. On the basis that $CoCl_2$ induces the hypoxic/ischemic condition, this study was designed to investigate 1) the mechanism of $CoCl_2$-induced apoptosis and 2) the effects of EGCG on $CoCl_2$-induced apoptosis in PC12 cells. PC12 is a cell line derived from rat pheochromocytoma and widely used as an *in vitro* model for investigating neuronal apoptosis, oxygen sensor mechanism, and neuronal differentiation[17].

Ⅱ. MATERIALS AND METHODS

Cell culture and treatment with $CoCl_2$ and EGCG

PC12 rat pheochromocytoma cells were maintained in RPMI 1640 medium containing 10% horse serum, 5% fetal bovine serum (Gibco BRL, USA) inactivated by heat and gentamycin (50 ㎍/ml, Gibco-BRL, NY, USA) under 5% CO_2 at 37℃. $CoCl_2$ was dissolved in distilled H_2O and sterilized through a 0.2 ㎛ filter before use. Cells were treated with each concentration of $CoCl_2$ for needed time in the same medium and EGCG was pretreated for 30 min before $CoCl_2$ treatment.

Cell viability assay by MTT assay

PC12 cells were plated onto 96 well plates and exposed to $CoCl_2$ alone or pretreated with different concentration of EGCG (Sigma ST. Louis, USA) for 30 min. After treatments, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) was added to the culture medium in respective times at a final concentration of 0.1 mg/ml and incubated at 37℃ for 4 hr. The reaction product of MTT was extracted in dimethylsulfoxide (DMSO) and optical density (OD) was spectrophotometrically measured at 570 nm with DMSO as a blank using a ELISA reader (ELx800uv, BIO Tek Instruments, Inc).

Flowcytometric analysis

After incubation with 150 ㎛M $CoCl_2$ alone or pre-

treated 100 μM EGCG for 30 min, cells were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). Cell pellets were resuspended in 0.2 ml of PBS containing 1 mg/ml propidium iodide (PI) and 0.1% Triton X-100. Fluorescence emitted from the PI-DNA complex was quantitated by FACScan cytometry (Becton Dickinson, San, CA).

Morphological change and DNA fragmentation

Morphological changes of apoptotic cells were investigated by propidium iodide (PI) stain. Cells plated in 60 mm dishes at a density of 1×10^6 was incubated at 18 hr and were treated with 150 μM $CoCl_2$. EGCG was added at 30 min before $CoCl_2$ treatment if necessary. The cells were stained with PI (Sigma, USA) and were observed under fluorescence microscope (Olympus, USA).

Oligonucleosomal fragmentation of genomic DNA was assessed using the Apopladder kit (TaKaRa Shuzo) according to the manufacture instructions. Each sample were electrophoresed at 80 V for 2 hr on 2.0% agarose gels. DNA bands were visualized under UV light after staining with ethidium bromide.

Determination of mitochondrial membrane potential and caspase activity

PC12 cells were grown on 60 mm dishes at 5×10^5 cells and treated with 150 μM $CoCl_2$. EGCG were pretreated at final concentration 1 hr before $CoCl_2$ treatment. The mitochondrial membrane potential ($\Delta\psi_m$) of cells was measured by staining with 5,5', 6.6'-tetrachloro-1,1',3,3'-tetrathylbenzimidazol carbocyanine iodide (JC-1, Molecular Probes). When mitochondria is polarized, JC-1 forms aggregates and emits orange fluorescence under 486 nm excitation. The orange and the green fluorescence were measured simultaneously by FACScan (Becton Dikinson, CA).

Caspase activity was assayed using the caspase-3, -9 activity assay kit (Calbiochem, San Diego, CA) and caspase-8 activity Kit (Santa Cruz, USA) according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR)

PCR were performed on a GeneAmp PCR system (Perkin-Elmer 2400). The following primer pairs were used: for Bax, 5'-GTTTCATCCAGGATCGAGCAG-3' (senseprimer) and 5'-CATCTTC TTCCAGATGGTGA-3' (antisense primer); for Bcl-2, 5'-CCTGTGG ATGACTGAGTACC-3' (sense primer) and 5'-GAGACAGCCAGGAGA AATCA-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, Upland, 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'-CGCCTGCTT CACCACCTTC-3' (antisense primer). The intensities of the obtained bands were determined using the NIH Scion Image Software.

Western blotting

Proteins solubilized in the lysis buffer (500 mM Tris-HCl, pH7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzamiden, 1 μg/ml Trypsin inhibitor) containing a cocktail of protease inhibitor (Complete, Mini. Roch, Germany). 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, UK) and incubated with the respective primary antibody. Rat monocloned antibodies against cytochrome *c* (Pharmingen, San Diego, CA) was applied. Blots were subsequently washed three times in TBS-T for 5 min and incubated with specific peroxidase-coupled secondary antibodies (anti-mouse IgG horseradish peroxidase [HRP], anti-Rabbit IgG-HRP, Sigma Aldrich). Bound antibodies were visualized using an enhanced chemiluminescent detection system (Amersham Pharmacia Biotech, UK).

Ⅲ. RESULTS

Effect of EGCG on cells viability in $CoCl_2$-treated cells

To asses cell viability for the time period, cell viability assay was performed by MTT assay. The cell viability was greatly reduced in dose- and time-dependent manners when PC12 cells exposed to $CoCl_2$ (Fig. 1A, 1B). The survival rate of PC12 cells was

about 65% when the cells were treated with 150 μM $CoCl_2$ for 8 hr. At the point of 24 hr after exposing to 150 μM $CoCl_2$, the percentage of cell viability was near zero. Treatment with different dose of EGCG for 30 min before PC12 cells were exposed to 150 μM $CoCl_2$ for 8 hr resulted in the cell viability being rescued in a dose-dependent manner (Fig. 2). The most effective dose of EGCG was 100 μM in promoting the cell viability in $CoCl_2$-treated PC12 cells.

Protective effects of EGCG on the $CoCl_2$-induced apoptosis

After treatment with 150 μM $CoCl_2$ for 8 hr, cells had great change in their shape observed by the a fluorescent microscopy after PI stain. The cells became swelling and apoptotic nuclei indicated by condensed nuclei and nuclear fragmentation appeared after exposure to 150 μM $CoCl_2$ for 8 hr whereas EGCG attenuated those in $CoCl_2$-treated PC12 cells (Fig. 3).

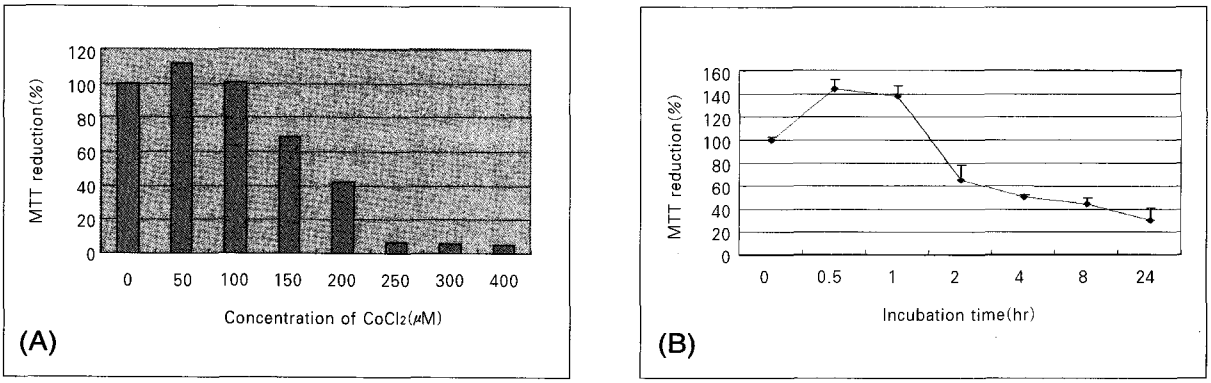


Fig. 1. The cell viability in PC12 cells treated with $CoCl_2$. PC12 cells were incubated in medium containing different concentrations of $CoCl_2$ (0, 50, 100, 150, 200, 250, 300 and 400 μM) for 8 hr (A), and treated with 150 μM $CoCl_2$ for time course (0, 0.5, 2, 4, 8 and 24 hr) (B).

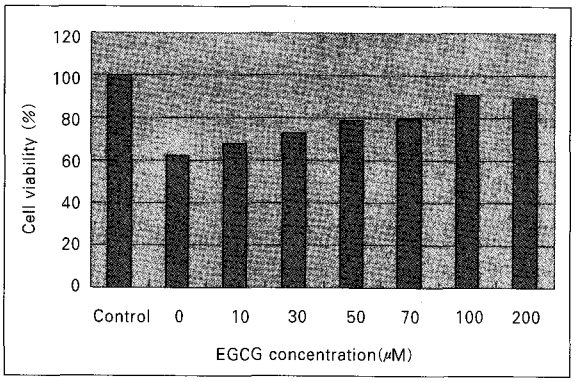


Fig. 2. Effects of EGCG on the survival of PC12 cells in 150 μM $CoCl_2$-treated conditions. PC12 cells were pretreated with increasing dose of EGCG (0, 10, 30, 50, 70, 100, 200 μM) in 100 μM $CoCl_2$-treated condition for 8 hr. Cell viability was determined by the MTT dye assay. Value from each treatment was indicated as a percent relative to the control (100%).

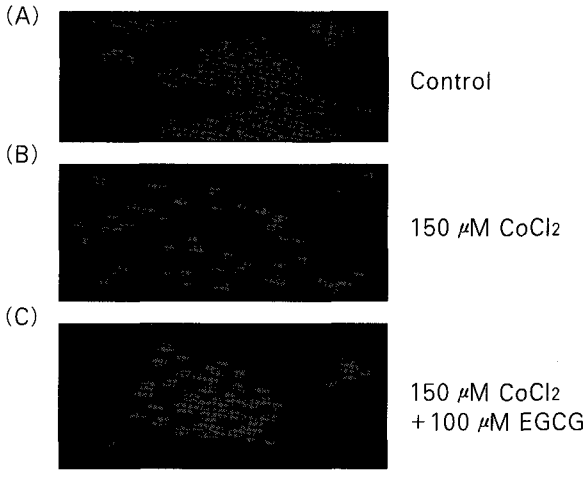


Fig. 3. Effect of EGCG on $CoCl_2$-induced morphological changes of PC12 cells.
PC12 cells incubated in the absence (A) or presence (B) of $CoCl_2$ only for 8 hr or pretreatment of EGCG for 30 min (C). The cells were stained with PI (1 μg/ml) and nuclear morphology was detected by fluorescence microscopy (×100).

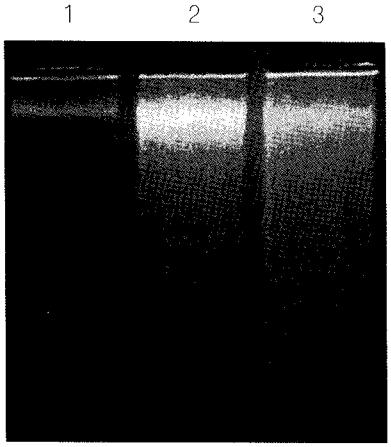
PC12 cells exposed to $CoCl_2$ for 8 hr demonstrated DNA fragmentation as a ladder pattern, meaning that $CoCl_2$ induced the apoptotic change. The laddering pattern in 150 μM $CoCl_2$-treated cells for 8 hr was slightly inhibited with the treatment of 100 μM EGCG for 30 min (Fig. 4). Furthermore, flow cytometry was performed to determine the apoptotic cells. When a number apoptotic cells were stained with a DNA-specific PI, a specific DNA peak, called as apoptotic peak (sub G1 peak) were appeared. Besides, pretreatment with 5 mM NAC, inhibited the apoptoic peak in $CoCl_2$-treated cells and EGCG suppressed in consistent with those of NAC effects (Fig. 5).

EGCG reduced the activity of caspases (-3, -8 and -9) in $CoCl_2$-treated cells

To determine whether caspases are involved in $CoCl_2$-induced apoptosis, specific caspases activities were assessed. $CoCl_2$ induced dramatic increase in all of caspase-3, -8 and -9 activities. The caspase activity were 2 folds in caspase-3, 3 folds in caspase-8 and about 1.5 folds in caspase-9 after exposure to $CoCl_2$(100 μM, 8 hr), compared with control respectively. Pretreatment with 100 μM EGCG for 30 min significantly attenuated the enhanced three caspases activities in $CoCl_2$-treated cells (Fig. 6, 7 and 8).

Effects of EGCG on the altered $\Delta\psi_m$ and cytochrome c release

To evaluate whether mitochondrial dysfunction is involved in $CoCl_2$-induced apoptosis, the loss of $\Delta\psi_m$ were measured and cytochrome c release from mitochondria into cytoplasm was detected. The JC-1 fluorescence intensities were shifted to left when PC12 cells were exposed to 150 μM $CoCl_2$ for 8 hr, indicating that $CoCl_2$ caused $\Delta\psi_m$ to be depolarized in PC12 cells. The left shift of JC-1 fluorescence after exposure to $CoCl_2$ was ameliorated under pretreatment of 100 μM EGCG or 5 mM NAC, a ROS scavenger for 30 min (Fig. 9). Cytoplasmic cytochrome c was upregulated when PC12 cells exposed to $CoCl_2$ (100 or



1. Control
2. 150 μM $CoCl_2$
3. 150 μM $CoCl_2$+ 100μM

Fig. 4. DNA fragmentation assay. DNA prepared from the cells treated for 8 hr with $CoCl_2$ only or pretreated with EGCG for 30 min before $CoCl_2$ treatment was electrophoresed and visualized with ethidium bromide.

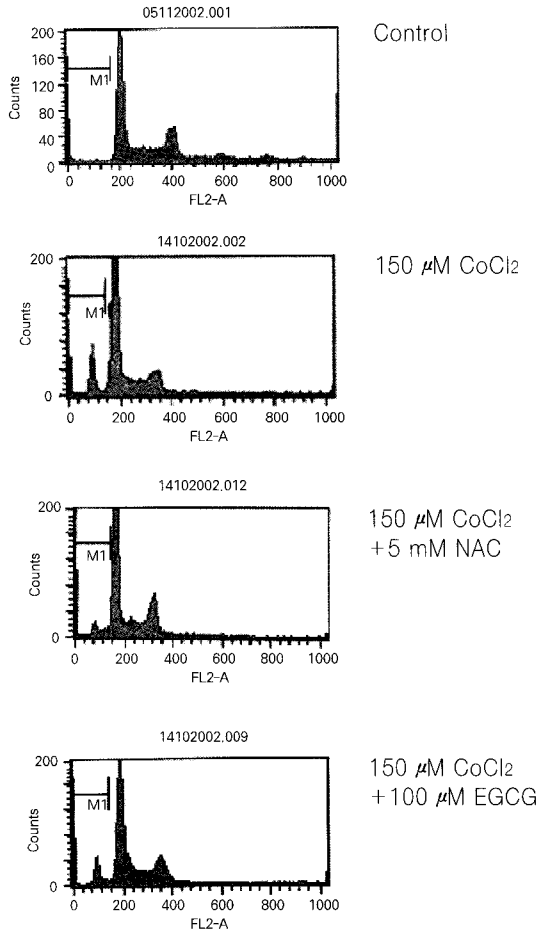


Fig. 5. Flow cytometric analysis. The cells were treated for 8 hr with $CoCl_2$ only or pretreated with EGCG or 5 mM NAC for 30 min before $CoCl_2$ treatment. After 24 hr incubation, DNA contents in cells were measured by FACScan.

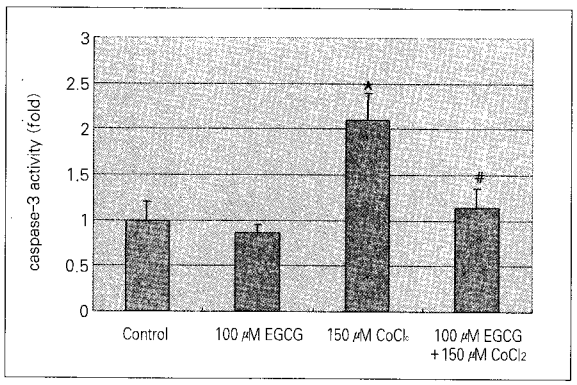


Fig. 6. Effect of EGCG on $CoCl_2$-induced caspase-3 activity in PC12 cells. Enzymatic activity of caspase-3 protease was determined by incubation of 50 ㎍ of total protein with DEVD-pNA substrate (200 μM).

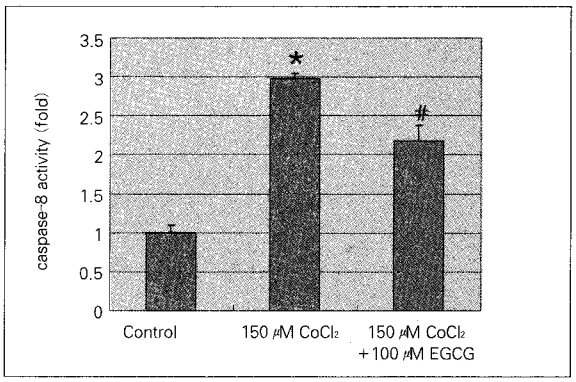


Fig. 7. Effect of EGCG on $CoCl_2$-induced caspase-8 activity in PC12 cells. Enzymatic activity of caspase-8 protease was determined by incubation of 50 ㎍ of total protein with IETD-AFC substrate (200 μM). $CoCl_2$(150 μM, 8 hr) enhanced caspase-8 activity and EGCG inhibited the increment in caspase-8 activity by $CoCl_2$ in PC12 cells. Each volume is mean ±SE. *P<0.05, compared with control. # P<0.05, compared with 150 μM $CoCl_2$-treated group.

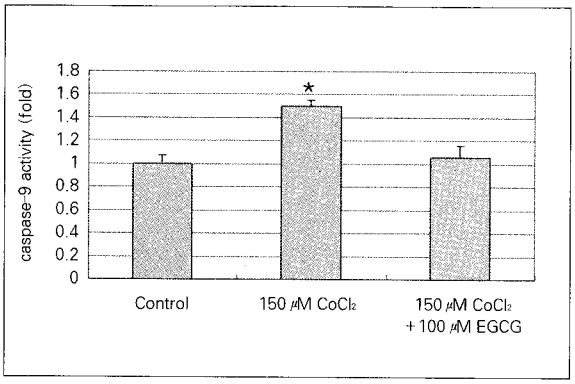


Fig. 8. Effect of EGCG on $CoCl_2$-induced caspase-9 activity in PC12 cells. Enzymatic activity of caspase-9 protease was determined by incubation of 50 ㎍ of total protein with LEHD-pNA substrate (200 μM). $CoCl_2$(150 μM, 8 hr) enhanced caspase-9 activity and EGCG inhibited the increment in caspase-9 activity by $CoCl_2$ in PC12 cells. Each volume is mean ±SE. *P<0.05, compared with control.

150 μM) for 30 min, meaning that cytochrom c were released from mitochondria into cytoplasm in $CoCl_2$-induced apoptosis, whereas EGCG suppressed the cytoplasmic cytochrome c (Fig. 10).

Effects of EGCG on Bax and Bcl-2 expression in $CoCl_2$-treated cells

To determine whether Bcl-2 family is an essential role in $CoCl_2$-induced apoptosis or not, Bax and Bcl-2 expression were observed using RT-PCR. Expression of Bcl-2, a cytochrome c releasing inhibitor from mitochondria into cytoplasm, was downregulated with treatment of 150 μM $CoCl_2$ for 8 hr. However, expression of Bax, a cytochrome c releaser, was not changed in $CoCl_2$-treated cells (Fig. 11). Downregulated expression of Bcl-2 was rescued when 100 μM EGCG was pretreated for 30 min before exposure to $CoCl_2$ (Fig. 11).

Ⅳ. DISCUSSION

Hypoxia-induced cell death is a major concern in various clinical entities such as ischemic disease, organ transplantation, and other disease. However, the mechanisms underlying the hypoxia-induced cell death and its treatment methods are not still defined.

In the present study, exposure of PC12 cells to $CoCl_2$ gradually reduced the cell viability in a dose- and time-dependent manners. Hypoxia-induced cell death can be partitioned into two basic forms, apoptosis and necrosis, on the basis of changes in morphology, enzymatic activity, ATP concentration and adjacent cellular effects[18,19]. In the present experiment, $CoCl_2$-treated cells demonstrated not only DNA fragmentation showing ladder pattern, but also morphologic changes such as cell swelling, condensed nuclei, and apoptotic nuclei. Moreover, when apoptotic cells stained with PI were measured using flow-

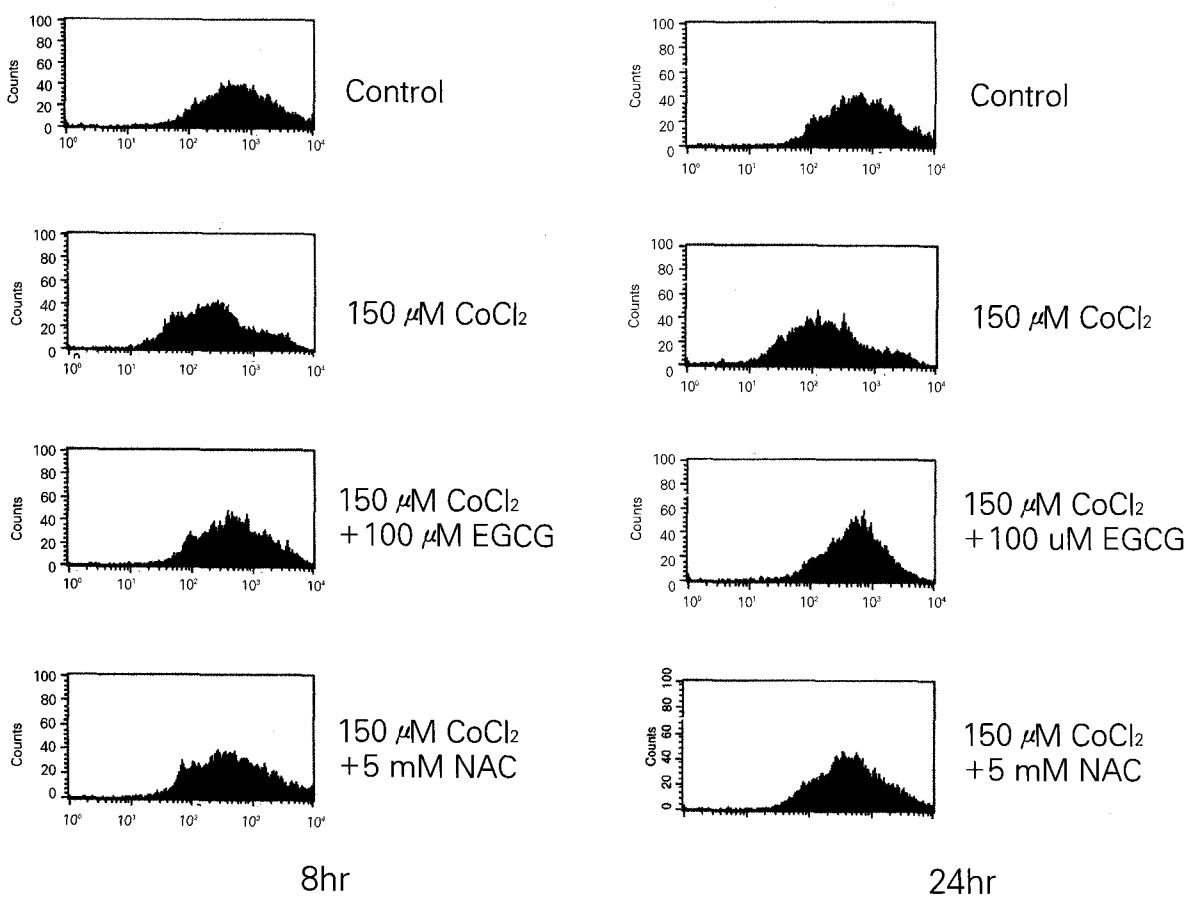


Fig. 9. Effect of EGCG on $CoCl_2$-induced mitochondrial membrane potential changes of PC12 cells. The mitochondrial membrane potential of cells was measured by staining with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyaniniodide.

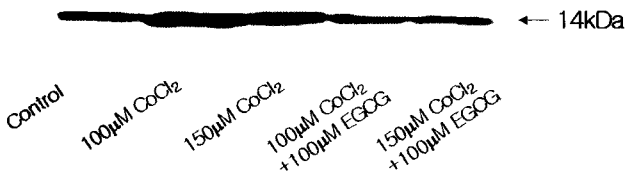
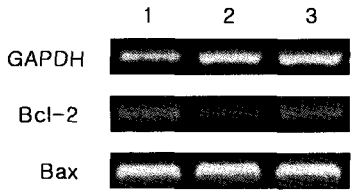


Fig. 10. Inhibition of $CoCl_2$-induced mitochondrial cytochrome c release by EGCG. Levels of cytochrome c were determined using immunoblot 8 hr afrer PC12 cells exposed to $CoCl_2$ with or without pretreatment of EGCG for 30 min.



1. Control
2. 150 μM $CoCl_2$
3. 150 μM $CoCl_2$+100 μM EGCG

Fig. 11. Effect of EGCG on Bcl-2 and Bax expression in $CoCl_2$-treated PC12 cells. The mRNA levels of Bax and Bcl-2 in $CoCl_2$treated PC12 cells for 8 hr. Hypoxia-induced cell death is a major concern in various clinical entities such as ischemic disease, organ transplantation, and other disease. However, the mechith or without pretreatment of EGCG for 30 min were determined by RT-PCR.

cytometry, sub-G1 peak called apoptotic peak appeared in $CoCl_2$-treated cells. Taken together, the present results suggested that $CoCl_2$ induces apoptosis of PC12 cells because gross nuclear changes and DNA fragmentation patterns were critical events of typical apoptosis. Also, $CoCl_2$-induced apoptosis is well documented to be driven through the production of ROS, subsequently resulting in activation of apoptotic machinery[2,16,20]. In the present study, NAC, a ROS scavenger, suppressed the apoptotic peak in $CoCl_2$-treated cells. From these results, it is speculated that $CoCl_2$-induced apoptosis is driven from the production of ROS.

Caspase-3 is a key and common protease in both mitoondria- and death receptor-dependent pathways and particularly important in neurons[21]. Previous studies have shown that caspase-3 is activated in response to various hypoxia and expressed in PC12 cells, indicating that caspase-3 may play a pivotal role in hypoxia-induced apoptosis in PC12 cells. Indeed, Zou *et al*[22] reported that caspase-3-like proteases are activated during the apoptotic cell death in $CoCl_2$-treated PC12 cells. Our results also showed that caspase-3 activity was upregulated in $CoCl_2$-treated cells, which consistent with that of Zou *et al*[22] These results suggest that $CoCl_2$-induced apoptosis involves a caspase-3 mediated mechanism. However, the upstream mechanism which activates caspase-3 has not been still elucidated in $CoCl_2$-induced apoptosis, even if some pathways are suggested in various hypoxia model. One possible mechanism for activating caspase-3 in $CoCl_2$-induced apoptosis is caspase-9 mediated process activated by cytochrom *c* released from the mitochondria, in concert with Apaf-1 and dATP, called as mitochondria mediated pathway. Mitochondria has been known to serve as a main target in various hypoxic/ ischemic model[3,23,24]. Although mitochondrial cytochrone *c* release and caspase-9 activation have been reported in focal and global ischemia models[17], no report has referred to change in mitochondria function. In the present study, 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. Moreover, the present study showed that a loss of $\Delta\psi_m$ was noted in $CoCl_2$-treated cells, supporting that there is a strong casual link between mitochondrial dysfunction and caspase activation in $CoCl_2$-induced apoptosis. Besides, recent reports have demonstrated that ROS leads to an induction of PT pore opening and a loss of $\Delta\psi_m$ under ischemia and related condition[25,26]. From the previous and present studies, it is assumed that ROS produced by $CoCl_2$ impaires mitochondrial function accompanied with loss of $\Delta\psi_m$ and cytochrome c release, subsequently activating caspase-9. One another passible mechanism for activating caspase-3 is caspase-8 mediated process activated by Fas and TNF receptor-1, called as death receptor-dependent pathway. In addition, it was recently known that Bid activated by caspase-8 is critical to ischemic neuronal apoptosis and focal cerebral ischemia[27]. From these previous reports, it is proposed a possibility that death receptor-dependent apoptosis pathway may be involved in caspase-3 activation in $CoCl_2$-induced apoptosis. However, there is no report examined the extrinsic pathway in $CoCl_2$-induced apoptosis. The present study shows the first evidence that death receptor-dependent pathway may be involved in $CoCl_2$-induced apoptosis. More interestingly, caspase-8 activity was upregulated and levels of caspase-8 activity was higher than those of caspase-9 activity when PC12 cells were exposed to $CoCl_2$ in the present study. These reslults suggest that caspase-8 mediated process plays an important role in $CoCl_2$-induced apoptosis, even if upstream and downstream of caspase-8 were unfortunately not examined in the present study. From these findings mentioned above, $CoCl_2$-induced apoptosis is likely to be mediated by both intrinsic and extrinsic apoptosis pathways.

The final question remains as to how ROS is produced or regulated by $CoCl_2$. Recent reports have showed that Bcl-2 family regulates the production of ROS and cytochrome c release from mitochondria in hypoxic/ischemic condition[28]. In general, the Bcl-2 family of proteins are well-characterized regulators of apoptosis, consisting of three distinct subfamily. The Bcl-2 subfamily contains antiapoptotic proteins such as Bcl-2 and Bcl-XL, which reduce cytochrome *c* release and a loss of $\Delta\psi_m$[29,30]. The Bax subfamily contains proapoptotic proteins such as Bax and Bak, which induce cytochrome *c* release and a loss of

$\Delta\psi_m$[29]. The Bcl proteins such as Bid, Bik and Bim are another subfamily of proapoptotic proteins, which are activated by caspase-8. Therefore, expression of Bcl-2 family was examined during $CoCl_2$-induced apoptosis in the present study to elucidate the involvement of Bcl-2 family in $CoCl_2$-induced apoptosis. In the present study, Bcl-2 mRNA was under expressed, but Bax mRNA was not changed in $CoCl_2$-treated cells. These findings suggest that Bcl-2 plays a critical role in $CoCl_2$-induced apoptosis. From these results, it is speculated that $CoCl_2$ suppressed Bcl-2 expression, subsequently enhancing the production of ROS, which activates extrinsic and intrinsic apoptotic pathway. However, roles of Bcl-2 family in the production of ROS may be a debate since ROS could conversely induce a decrease in Bcl-X_L mRNA[31]. and release of cytochrome *c* triggers accumulation of ROS[20].

On the other hands, EGCG has been known to display a potent antioxidant property and its antioxidant effects have been extensively studied. Previous several *in vitro* and *in vivo* reports have showed that EGCG has a neuroprotective effect against neuronal injury and hippocampal neuronal damage after transient global ischemia[32]. Besides, green tea extract has been demonstrated to protect brain, liver, and kidney from lipid peroxidation injury. More recently, several reports have documented that EGCG attenuates β-amyloid-induced neurotoxicity in cultured hippocampal neurons and reduces 6-hydroxydopamine-induced apoptosis in PC12 cells[14,33]. However, there is no previous report reffered to the neuroprotective effects of EGCG against $CoCl_2$-induced neuronal apoptosis. The present study is the first report documented that EGCG has a neuroprotective effect against $CoCl_2$-induced neuronal apoptosis. In the present study, EGCG remarkably reduced the cell death by $CoCl_2$ in PC12 cells. Moreover, EGCG inhibited DNA fragmentation and morphologic changes such as cell swelling and condensed nuclei in $CoCl_2$-treated cells. In addition, EGCG attenuated the apoptotic peak in $CoCl_2$-treated cells. These present results suggest that EGCG has a neuroprotective effects by inhibiting the neuronal apoptosis in $CoCl_2$-induced neurotoxicity. One of possible antiapoptotic mechanisms of EGCG may be its direct scavenging of ROS produced by $CoCl_2$, since polyphenols such as EGCG can scavenge different kinds of ROS and organic free radical, for example, superoxide anion, hydroxyl radicals, and lipid free radical[33]. In the present study, EGCG attenuated the increments in caspase-3, -9 and -8 activity by $CoCl_2$. Furthermore, EGCG ameliorated a loss of $\Delta\psi_m$ and cytochrome c release from mitochondria. These results support that EGCG prevents $CoCl_2$-induced neuronal cell death by inhibiting apoptosis. In addition, EGCG ameliorated the decrement in Bcl-2 expression, suggesting that genes associated with apoptosis are not altered after EGCG treatment. However, it is unknown that EGCG directly or indirectly regulates Bcl-2 expression through scavenging ROS. Recently, it was reported that green tea polyphenols can pass through the brain-blood barrier to exert neuroprotective effects, since EGCG was present in brain, liver and plasma 60 min after the administration of green tea polyphenols. Therefore, we speculate that administration of green tea prevents hypoxia-induced neurotoxicity by scavenging ROS produced by hypoxia.

Ⅴ. CONCLUSIONS

The present results suggest that $CoCl_2$-induced apoptosis is mediated by both mitochondria- and death receptor- dependent pathways and EGCG prevents $CoCl_2$-induced neuronal death through inhibition of apoptosis in PC12 cells.

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Abstract

PC12 세포에서 $CoCl_2$ 유발 세포자멸사에 대한 epigallocatechin-gallate의 역할

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신경세포자멸사는 저산소 및 허혈환경에서 일어나며 이러한 세포죽음은 reactive oxident species (ROS) 생성을 동반함이 알려져있다. 그러나, 저산소 및 허혈환경에서 일어나는 세포자멸사의 기전 및 그 치료방법은 아직 정립되어 있지 않다. $CoCl_2$는 ROS를 생성하는 등 저산소환경과 유사한 조건을 초래하는 것으로 알려져 있다. Epigallocatechin gallate (EGCG)는 녹차의 polyphenol 성분으로서 세포성장과 죽음에 다양한 약리학적 효과를 나타냄이 알려져 있다. 본 연구는 PC12 세포에서 $CoCl_2$에 의한 세포자멸사기전을 밝히고 이에 미치는 EGCG의 효과를 조사하는데 목적이 있다. Cell viability는 MTT 측정으로 조사되었고, DNA fragmentation은 DNA laddering으로 조사되었다. Bcl-2와 Bax 발현 정도는 RT-PCR로, caspase-3와 -9의 활성은 spectrophotometer, caspase-8의 활성은 flow cytometry에 의해 측정되었다. 미토콘드리아에서 세포질로 분비된 cytochrome *c*는 western blot으로, 분해된 DNA 양과 미토콘드리아 세포막전위($\Delta\psi_m$)는 FACScan으로 조사되었다.

$CoCl_2$ 투여로 PC12 세포수는 용량 및 시간 의존형태로 감소하였고, genomic DNA fragmentation이 발생하였다. $CoCl_2$ 투여로 야기된 cell viability의 감소와 DNA fragmentation은 EGCG 전처치에 의해 억제되었다. $CoCl_2$는 세포용적팽창과 condensed nuclei 같은 형태적 변화를 일으켰으며, apoptotic peak, $\Delta\psi_m$ 감소 및 cytochrome c 유리를 야기하였다. EGCG는 $CoCl_2$에 의한 세포형태변화, apoptotic peak, $\Delta\psi_m$ 소실 및 cytochrome *c* 유리를 억제시켰다. $CoCl_2$는 Bcl-2 발현을 감소시켰지만, Bax 발현에는 영향을 미치지 않았다. EGCG는 $CoCl_2$에 의해 야기된 Bcl-2 발현 감소를 억제시켰다. $CoCl_2$는 caspase-3, -8, 그리고 -9의 활성을 증가시켰으며, EGCG는 그 정도를 감약시켰다. ROS 제거제인 NAC (N-acetyl-cysteine)은 EGCG의 결과와 같은 양상으로 $CoCl_2$에 의한 세포자멸사를 억제시켰다.

본 실험결과는 PC12 세포에서 $CoCl_2$가 미토콘드리아 의존 및 death receptor 의존 기전으로 세포자멸사를 일으키며, EGCG는 세포자멸사기전을 억제시킴으로 신경보호기능을 가짐을 시사하였다.

주요어 : $CoCl_2$, 세포자멸사, EGCG, 미토콘드리아 의존기전, Death receptor의존기전