Master's Thesis

Intracellular Pathway and Neuroprotective Effect of *Carpinus tschonoskii* MAX on 6-Hydroxydopamine-Induced Death of PC12 Cells

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6-Hydroxydopamine에 의한 PC12 세포 사멸 기전 및 개서어나무의 신경보호 효과

지도교수 강 희 경

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이 논문을 의학 석사학위 논문으로 제출함

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ABSTRACT

The biochemical mechanisms involved in dopaminergic neuron loss in Parkinson's disease are not clearly defined. Recent studies reported that neuronal cell death is associated with Calpain/Cdk5-mediated hyperphosphorylation of myocyte enhancer factor 2 (MEF2), a critical transcription factor for neuronal survival, in MPTP model. We therefore investigated the possible involvement of Calpain/Cdk5-mediated MEF2D downregulation for 6-OHDA-induced death of PC12 cells.

In this study, we found that 6-OHDA significantly decreased MEF2D level in a concentration/time-dependent manner in PC12 cells. We identified that the decrease MEF2D by 6-OHDA in PC12 cells was increased by Calpain inhibitor PD151746 and Cdk5 inhibitor Roscovitine, respectively. We evaluated that 6-OHDA-induced the degradation of MEF2D is dependent on both Calpain and Cdk5 activity. On the other hand, we analyzed the phosphorylation state of several MAP kinase pathway and PI3/Akt pathway, a central role in cell death and survival. After the PC12 cells were treated with 6-OHDA, the phosphoylation of ERK1/2, JNK and p38 was increased, whereas the phosphoylation of Akt was decreased in a time-dependent manner compared to the control. These results suggest that MEF2D downregulation, MAP kinase activation and Akt inactivation plays a central role in PC12 cell death by 6-OHDA. Roscovitine, an inhibitor of Cdk5, increased the phosphorylation of ERK1/2 and JNK, while the phosphorylation of p38 was decreased. These results suggest that a relationship between Cdk5 and MAP kinase signals may play important roles for determining either survival or death of neuronal cells. We also examined the neuroprotective effects of *Carpinus tschonoskii* MAX on 6-OHDA-induced death of PC12 cells. *C. tschonoskii* attenuated PC12 cell death induced by 6-OHDA by the following mechanisms: it inhibited the generation of oxidative stress (ROS and NO); it recovered MEF2D levels; it

decreased the activities of the ERK 1/2 and JNK pathways; it reduced the expression of Bax and increased those of Bcl-2, procaspase-3 and PARP. Collectively, these results provide evidence that a *C. tschonoskii* extract prevents PC12 cell death induced by 6-OHDA, and that it may provide the tools as the therapeutic strategies in neurodegenerative disorder including Parkinson's disease.

Key word: Parkinson's disease, 6-OHDA, PC12 cell, MEF2D, Cdk5, MAP kinase, PI3/Akt pathway, *Carpinus tschonoskii* MAX

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Ⅰ. INTRODUCTION

1. Background

1.1. Parkinson's disease (PD)

Parkinson's disease (PD) is currently the most common degenerative disorder of the aging brain after Alzheimer's disease (Jordi Bove *et al*., 2005). PD is a neurodegenerative disorder characterized by motor symptoms, including tremor, muscle rigidity, paucity of voluntary movements and postural instability (Sandyk R *et al*., 1992; Lang and Lozano, 1998). The pathological hallmark of PD involves the loss of the nigrostriatal dopaminergic pathway, resulting in a marked impairment of motor control (David *et al*., 2001). However, the pathogenic processes in PD are not fully understood. Although its etiology remains unknown, oxidative stress is thought to be a critical mediator of damage in PD (Przedborski, 2005; Jenner and Olanow, 1996). The death of dopaminergic neurons by oxidative stress might include altered activities of survival signaling factors, including myocyte enhancer factor 2 (MEF2) (Mao et al., 1999), MAP Kinase pathways (Veeranna *et al*., 2000) or the PI3K/Akt pathway (Shimoke *et al*., 2001; Greggio *et al*., 2007).

1.2. Neurtotoxin: 6-Hydroxydopamine (6-OHDA)

6-Hydroxydopamine (6-OHDA) was the first dopaminergic neurotoxin discovered, and it is the most commonly used neurotoxin *in vivo* and *in vitro* (Blum, D *et al*., 2001). 6-OHDA is a selective catecholaminergic neurotoxin used to produce PD models (Blum, D *et al*., 2001). 6-OHDA induces nigrostriatal dopaminergic damage via the generation of ROS (Heikkila and Cohen, 1971) and inhibition of mitochondrial complex 1 (Glinka and Youdim, 1995;

Glinka *et al*., 1996). However, the detailed molecular mechanisms for 6-OHDA-induced apoptosis remain to be elucidated.

1.3. Mechanisms of dopaminergic neuron cell death

Recent studies show that reduced MEF2 transcriptional activity during neuronal apoptosis is associated with Calpain/Cdk5-mediated hyperphosphorylation of MEF2 (Mao and Wiedmann, 1999). Cdk5-induced phosphorylation of MEF2D at the Serine 444 inactivating site plays an essential role in dopaminergic neuron death induced by MPTP (Smith *et al*., 2006) and glutamate (Tang *et al*., 2005). Activated caspase induces cleavage of p-MEF2D (phosphorylation of Ser 444 residue) and protease-mediated degradation of MEF2D, resulting in the inhibition of MEF2-dependent survival gene expression and neuronal cell death (Li *et al*., 2001; Okamoto *et al*., 2000). These studies suggest that down regulation of MEF2 could be involved in the death of cortical neurons induced by MPP^+ and glutamate (Gong *et al*., 2003; Tang *et al*., 2005; Smith *et al*., 2006). Nevertheless, how this pathway underlies PC12 cell death due to 6-OHDA remains unknown.

1.3.1. Cyclin-dependent kinase 5 (Cdk5) in PD pathology

Cdk5 is a member of the cyclin-dependent kinase (Cdk) family of Serine/Threonine kinases (Hisanaga *et al*., 2003). In contrast to the classical cell cycle-related CDKs, cdk5 is predominantly involved in the regulation of neuronal signaling (Kwon and Tsai, 2000) and requires associations with its regulatory partners, p35 or p39, in order to be active (Hisanaga *et al*., 2003; Ko *et al*., 2001). Cdk5 has been implicated in the regulation of several neuronal processes, including neurodevelopment (Ohshima *et al*., 1996; Ko *et al*., 2001), axonal transport (Julien and Mushynski, 1998), synaptic activity (Rosales *et al*., 2000) and dopamine signaling (Nishi *et al*., 2002; Chergui *et al*., 2004). Although Cdk5 is involved in

the regulation of normal physiological functions, recent correlative evidence suggests that Cdk5 may be the cause of neuronal death signals (Shelton and Johnson, 2004; Smith *et al*., 2004).

Under neurotoxic conditions, Calpain cleaves p35. This produces p25, which alters Cdk5 substrate specificity and results in abnormal activity. Generation of p25 is thought to increase Cdk5-dependent phosphorylation of various survival factors, such as MEF2D, which are pathological markers of Parkinson's disease and Alzheimer's disease (Mao and Wiedmann, 1999; Baumann *et al*., 1993; Lund *et al*., 2001; Smith and Tsai, 2002; Cruz *et al*., 2003). This is consistent with earlier evidence of over activation of Cdk5 in postmortem PD patients (Mouatt-Prigent A *et al*., 1996). However, the mechanism by which Cdk5 regulates neurodegeneration is not clearly defined.

1.3.2. Myocyte enhancer factor 2 D (MEF2D) and Cdk5

In mammals, Myocyte enhancer factor 2 (MEF2) is a family of 4 transcription factors: isoforms MEF2A, B, C and D. MEF2 was first identified in 1989 for its DNA binding activity in the promoters of several muscle-specific genes (Braun, Tannich *et al*., 1989; Gossett, Kelvin *et al*., 1989). The MEF2 family is highly expressed in developing neurons during dendritic maturation and synapse formation (Lyons *et al*., 1995; McKinsey *et al*., 2002; Shalizi and Bonni, 2005). MEF2 transcription factors play critical roles in several key intracellular signaling pathways, including neuronal survival and apoptosis (Black and Olson, 1998; Mao *et al*., 1999; Mao and Wiedmann, 1999; Okamoto *et al*., 2000; Li *et al*., 2001; Gong *et al*., 2003; Liu *et al*., 2003). More recent studies have shown that MEF2 regulates excitatory synapses in hippocampal and cerebellar granule neurons (Flavell *et al*., 2006; Shalizi *et al*., 2006, 2007). In addition to controlling synaptogenesis, MEF2 transcription factors may influence neuronal differentiation by supporting the survival of newly formed

neurons (Mao *et al*., 1999; Okamoto *et al*., 2000; Gaudilliere *et al*., 2002; Linseman *et al*., 2003). Consequently, MEF2 activity appears to be regulated in part by the phosphorylation of Cdk5 (Ser408 in MEF2A, Ser444 in MEF2D), which inhibits MEF2 activity (Flavell *et al*., 2006; Gong *et al*., 2003; Mao and Wiedmann, 1999).

1.4. Mitogen-Activated Protein Kinase (MAPK) Pathway

Mitogen-activated protein kinase (MAPK) signaling and the phosphoinositide 3-kinase (PI3K)/Akt pathway play a central role in cell death and survival (Chong *et al*., 2005; Veeranna *et al*., 2000; Brunet *et al*., 2001). Oxidative stress involves several intracellular signaling pathways, one of which may include the stress-activated protein kinases. c-Jun N-terminal kinases (JNK) (Pantano *et al*., 2003; Mielke *et al*., 2000) and p38 MAP kinases (Junn and Mouradian, 2001) are activated in response to neurotoxin and induce neuronal apoptosis. Interestingly, phosphorylated forms of SAPK/JNK, p38 and ERK1/2 are expressed in postmortem brains of PD (Ferrer *et al*., 2001) and AD (Knowles *et al*., 1999).

1.5. PI3K/Akt Pathway

Phosphoinositide 3-kinase (PI3K)/Akt is a reported neuronal survival factor (Saito *et al*., 2004; Brunet *et al*., 2001; Sonoda *et al*., 1999; Dudek *et al*., 1997). Akt is a key molecule in growth factor signaling pathways (Crowder and Freeman, 1998; Johnson-Farley *et al*., 2006). It mediates neuronal survival in both development and disease, including resistance against oxidative insults in the brain (Noshita *et al*., 2001; Li *et al*., 2003; Chong *et al*., 2005). Thus, inactivation of the PI3K/Akt pathway might induce neuronal apoptosis.

1.6. Signaling Pathways Involving the Cdk5/p35 Pathway

Previous studies reported that the Cdk5 pathway might be involved in regulating the MAP

kinase cascade. Sharma (2002) showed that NGF induced activation of the ERK1/2 pathway, which caused activation of the EGR-1 transcription factor and upregulation of p35. Cdk5/p35 induces phosphorylation of MEK1 at Thr286, which promotes feedback for down-regulating the ERK1/2 pathway (Harada *et al*., 2001; Sharma *et al*., 2002; Zheng *et al*., 2007). Cdk5 directly phosphorylates JNK3 at Thr131 to inhibit its kinase activity in HEK293T cells after UV irradiation (Li *et al*., 2002). Also, Nikolic (1998) showed that Cdk5 and p35 associated with the Rac pathway, upstream of JNK and p38 signaling, in a GTP-dependent manner (Nikolic *et al*., 1998). There may be a link between Cdk5 and stress-activated protein kinase signaling, such as JNK and p38, involved in neuronal apoptosis (Zhang *et al*., 2002; Kyriakis *et al*., 1994). Collectively, these observations suggest that there may be an interaction between Cdk5 and MAP kinase pathways in regulating neuronal survival and apoptosis.

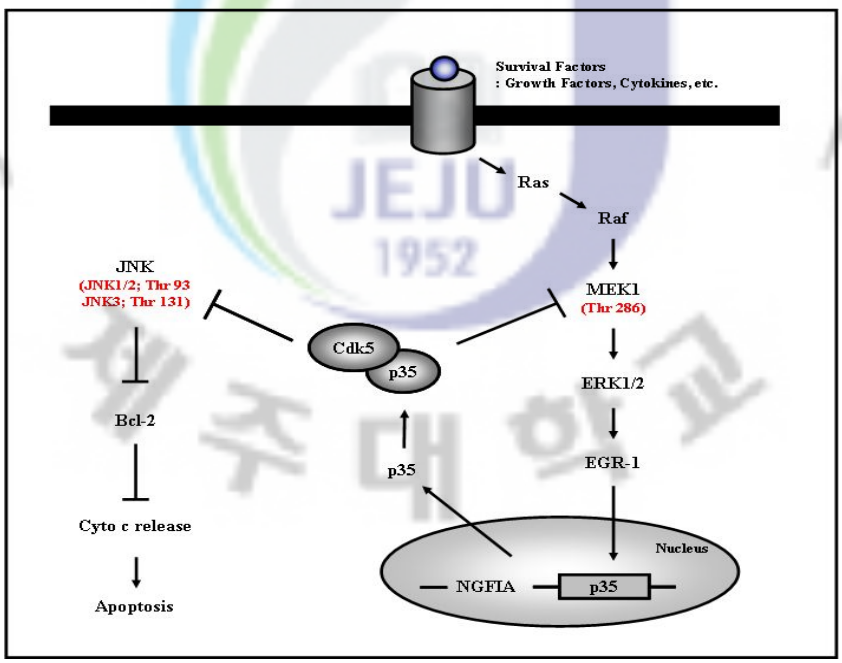


Figure 1. Model for how Cdk5 regulates MAP kinase in PC12 cells to regulate neuronal survival and apoptosis. This is based on previously reported data (Harada et al., 2001; Sharma et al., 2002; Zhang et al., 2002).

1.7. *Carpinus tschonoskii* MAX

Genus *Carpinus* comprises nearly 40 species, which are widely distributed throughout eastern Asia, Europe and North/Central America (Rehder, 1927; Jones and Luchsinger, 1986; Hillier, 1988). However, only 5 species inhabit Korean: C. *tschonoskii, C. laxiflora, C. cordata, C. turczaninowi,* and *C. coreana* (Lee *et al.,* 1989). Chang and Jeon (2007) reported the isolation of flavonoid compounds from the leaves of *Carpinus*, which mainly consisted of mono- and di-galactosides of the flavonols (myricetin, quercetin and kaempferal) and the flavones (apigenin and luteolin). Recent studies have shown cytoprotective activities of a *Carpinus tschonoskii* methanol extract against H_2O_2 induced oxidative stress (Zhang *et al*., 2007). The MeOH extract of *Carpinus tschonoskii* exhibited ROS scavenging activity, which promoted V79-4 Chinese hamster lung fibroblast viability against H_2O_2 induced oxidative stress (Zhang *et al*., 2007). However, possible neuroprotective effect of *Carpinus tschonoskii* EtOH extract and its mechanism in a PD model remain unknown.

2. Purpose

The molecular basis for neuronal death in the context of PD pathogenesis remains unknown. In the present study, we investigated if 6-OHDA-induced death of PC12 cells might involve the downregulation of MEF2D. We used an inhibitor of Cdk5, roscovitine, to determine if there was a link between Cdk5 and MAP kinase signals in 6-OHDA-induced death of PC12 cells. We also examined any neuroprotective effect of *Carpinus tschonoskii* MAX on dopaminergic neuronal cell death induced by 6-OHDA in PC12 cells.

Ⅱ. MATERIALS AND METHODS

1. Reagents

The following reagents were obtained commercially: 6-hydroxydopamine (6-OHDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Hoechst 33342 were from Sigma (St. Louis, MO, USA); Monoclonal anti-myocyte enhancer factor 2 D (MEF2D) was from BD Biosciences. Rabbit polyclonal anti-Bax, anti-Bcl-2, anti-cleaved caspase-3, anti-p44/42, anti-phospho-p44/42, anti-p38, anti-phospho-p38, anti-Akt, anti-phospho-Akt, anti-SAPK/JNK and anti-phospho-SAPK/JNK were from Cell signaling Technology; Rabbit polyclonal anti-caspase-3 and anti-poly (ADPribose) polymerase (PARP) were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal β-actin was from Sigma (St. Louis, MO, USA); HRP-conjugated goat anti-rabbit and horse anti-mouse IgGs were from Vector (Vector Laboratories, Burlingame, USA); PD 151746 (Calpain Ⅰ inhibitor), Roscovitine (Cdk5 inhibitor) were from Calbiochem; Aprotinin, leupeptin, Nonidet P-40 were from Roche (Roche Applied Science, Indianapolis, IN); West-zol enhanced chemilumin, Western blotting detection reagent was from Intron (Intron Biotechnology, Korea).

2. Cell cultures

PC12 cells, a rat pheochromocytoma cell line, were supplied by KCLB (Korea Cell Line Bank). PC12 cells were incubated in RPMI 1640 medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL, USA) at 37°C under an atmosphere of 95% air and 5% CO_2 (Batistatou and Greene, 1993). All experiments used cells plated at a density of 1.0 x 10^5 cells/㎖.

3. Measurement of cell viability

Viable cells are able to convert the soluble dye MTT to insoluble blue formazan crystals. For the determination of cell viability, MTT assay was performed as described previously with modification (Scudiero, D. A., 1988). PC12 cells were grown on 24-well plates at a density of 1.0 x 10^5 cells/㎖. After the cells were attached for 18 hr, they were treated with 6-OHDA at different concentrations for 24 hr. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the cells at a final concentration of 250 μM and further incubated at 37 ℃ with 5% CO_2 for 4 hr to produce a dark blue formazan product by MTT reduction. Media were aspirated and formed formazan crystals were dissolved in DMSO (Aresco, USA). The absorbance of each well was measured using a microplate reader (Amersham Pharmacia Biotech, NY, USA) at 540 nm excitatory emission wavelength. The viability of PC12 cells was determined as a percent of inhibition due to reduced absorbance compared to the untreated controls.

4. Evaluation of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were determined using the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Rosenkranz *et al*., 1992). This molecule is cleaved intracellularly by esterases to nonfluorescent 2',7'-dichlorofluorescin (DCFH), which leads to the fluorescence compound 2',7'-dichlorofluorescein (DCF) upon oxidation by ROS. PC12 cells (1.0 × 10^5 cells/㎖) were seeded in 6-well plates. The cells were treated with 6-OHDA for 30 min, 1 h, 2h and 3h, then washed in PBS. The cells were incubated with 50 μM DCFH-DA for 20 min at 37 °C with 5% CO_2 in the dark. PC12 cells were washed twice with PBS and fluorescence was monitored by COULTER® EPICS® XL™ Flow Cytometer (Coulter, Miami, FL, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

5. Measurement of nitric oxide (NO) production

After pre-incubation of PC12 cells (1.0×10^5 cells/㎖) for 18 h, the cells were treated with varying concentrations of 6-OHDA for the indicated times. Nitrite in culture supernatants was measured by adding 100 ㎕ of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 ㎕ samples of medium. All measurements were performed in triplicate. The concentration of NO_2^- was determined from a standard curve prepared using $NaNO_2$.

6. Morphological analyses

Changes in nuclear morphologies of apoptotic cells were investigated by labeling the cells with the nuclear stain Hoechst 33342 and fluorescent microscopy. Briefly, the PC12 cells pre-plated in 24-well plate (1.0×10^5 cells/ml) were treated with 250 μM 6-OHDA for different times. Then the cells were stained with Hoechst 33342 (5 ㎍/㎖), and observed using fluorescence microscopy (Ⅸ-71, Olympus, Japan).

7. DNA fragmentation

PC12 (1.0×10^5 cells/㎖) cells were pre-incubated for 18 hr, and then treated with 250 μM 6-OHDA for the indicated times. After incubation, the cells were collected and washed twice with cold-PBS. DNA was extracted using a Promega Wizard® Genomic DNA Purification Kit (Promega, WI, USA). DNA samples (10 μL) were electrophoresed on a 1.2% agarose gel in 450 mM Tris borate-EDTA buffer, pH 8.0. DNA was observed under UV transiluminator (Spectronics Corporation Westbury, NY, USA).

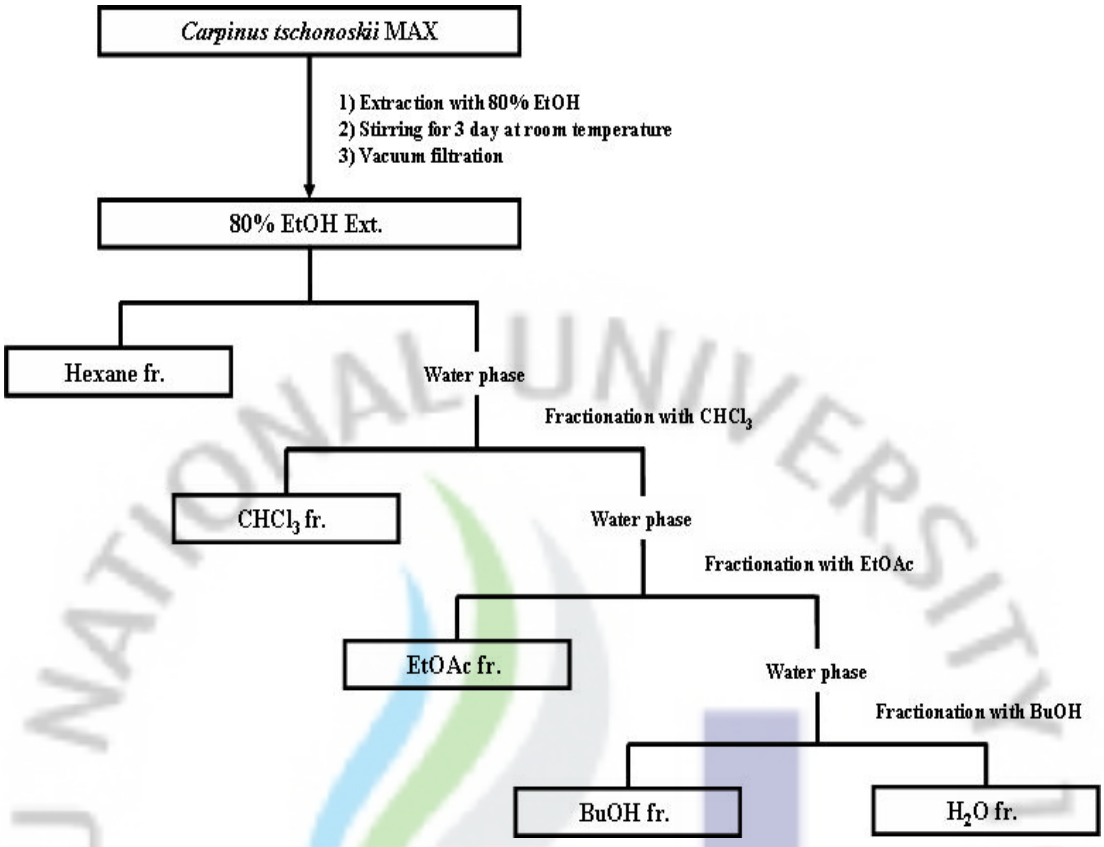
8. Western blot analysis

PC12 (1.0×10^5 cells/㎖) cells were pre-incubated for 18 hr, and then treated with varying

concentrations of 6-OHDA for the indicated times. After incubation, the cells were harvested and washed twice with cold-PBS. The cells were lysed in a lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM/l EDTA, 1 mM EGTA, 1 mM NaVO3, 10 mM NaF, 1 mM dithiothreitol, 1 mM Phenylmethylsulfonylfluoride, 25 ug/ml aprotinin, 25 ug/ml leupeptin, 1 mM DTT, 1% Nonidet P-40) to obtain whole cell protein and kept on ice for 30 min. The cell lysates were centrifuged at 15,000 rpm at 4 °C for 15 min. Supernatants were stored at -20 °C until analysis. Protein concentration was determined by Bradford method (Bradford, 1976). Equal amounts of protein were loaded onto a SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel. After electrophoretic separation, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-rad, HC, USA) with a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)) at 120 V for 1.5 hr. After blocking with 1% bovine serum albumin (BSA) in TBS-Tween (TBS-T) (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated with specific primary antibodies. The primary antibodies used in this study were as follows (see Table 1). Primary antibody incubation was followed by washing in 0.1% Tween-20 TBS solution and then incubating with a secondary HRP antibody (1:5000; Vector Laboratories, Burlingame, USA) at room temperature. A chemiluminescence reaction (ECL, Intron Biotechnology, Korea) was used to visualize protein bands on X-ray films (AGFA, Belgium). All blots were probed with β-actin to confirm that equal amounts of protein were loaded.

9. Statistical analysis

Student's t-test was used to compare values of experimental and control groups. Results are expressed as means ± standard deviation (SD) of at least 3 independent experiments performed in triplicate. P-values < 0.05 were considered statistically significant.



Scheme 1. Procedure for solvent extraction of *Carpinus tschonoskii* MAX

Antibody	Origin	Company
Myocyte enhancer factor 2 (MEF2D)	mouse monoclonal	BD Biosciences, USA
Bax	rabbit polyclonal	Cell signaling Technology
Bcl-2	〃	〃
Caspase-3	〃	〃
p44/42	〃	〃
phospho-p44/42	〃	〃
p38/MAPK	〃	〃
phospho-p38/MAPK	〃	〃
Akt	〃	〃
phospho-Akt	〃	〃
SAPK/JNK	〃	〃
phospho-SAPK/JNK	mouse monoclonal	〃
Procaspase-3	rabbit polyclonal	Santa Cruz Biotechnology
poly-(ADP-ribose) polymerase (PARP)	〃	〃
β-actin	mouse monoclonal	〃

Table 1. Antibodies used in Western blot analysis.

Ⅲ. RESULTS

1. Intracellular pathways involved with 6-hydroxydopamine-induced death of PC12 cells

1.1. 6-OHDA-induced death of PC12 cells

PC12 cells were treated with 6-OHDA at final concentrations of 50, 100, 150, 200 and 250 μM. After incubation for 24 h, PC12 cell viability was measured by the MTT assay. As shown in Fig.2, PC12 cell death was significantly increased by 6-OHDA in a dose-dependent manner. We determined that 250 μM 6-OHDA induced about 50 % cell death after 24 h, and this concentration was used in subsequent experiments (Fig. 2).

1.2. 6-OHDA-induced apoptosis of PC12 cells

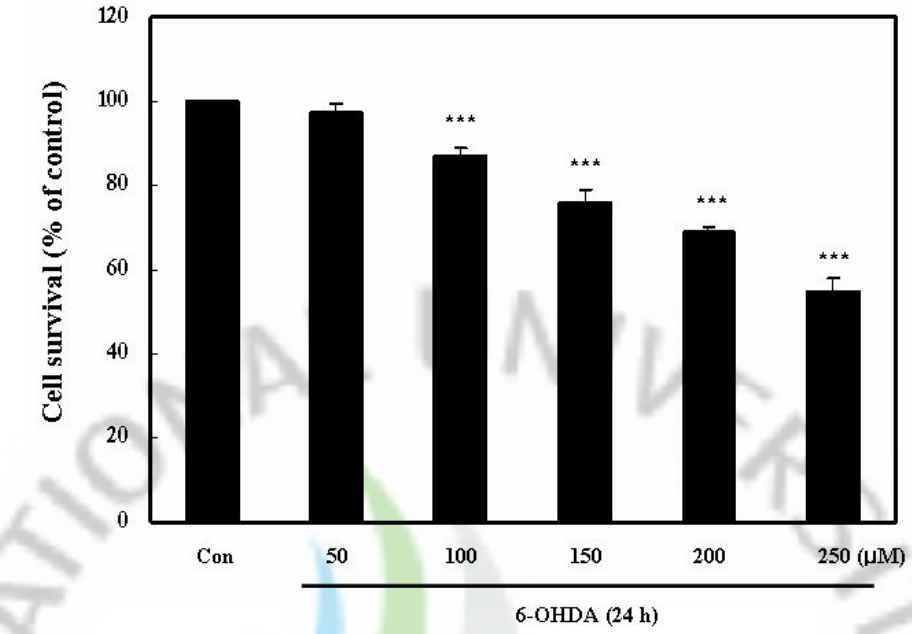
To examine nuclear morphology changes during PC12 cell death induced by 6-OHDA, Hoechst 33342 nuclear stain was used. Exposure to 250 μM 6-OHDA caused nuclear chromatin condensation, suggesting increased apoptosis (Fig. 3A). Consistent with this, 6-OHDA also increased DNA fragmentation in a time-dependent manner (Fig. 3B). To elucidate which apoptotic signals were triggered by 6-OHDA, we examined the expressions of Bax and Bcl-2, the activation of caspase-3 and the cleavage of poly-(ADP-ribose) polymerase (PARP). 6-OHDA increased Bax expression, while Bcl-2 expression was decreased after treatment for 3 h (Fig. 4). Caspase-3 is activated by multiple proteolytic cleavages of its 32 kDa precursor to generate an enzymatically active p12/p17 complex, which has been used to monitor the activation of caspase-3 (Nicholson *et al.*, 1995). Increased caspase-3 cleavage is associated with increased poly (ADP-ribose) polymerase (PARP) cleavage, a downstream substrate of caspase-3. 6-OHDA increased the cleavage of

caspase-3, whereas the PARP level decreased in a time-dependent manner (Fig. 4).

1.3. 6-OHDA generates reactive oxygen species (ROS) and nitric oxide (NO)

In neurons, the apoptotic pathway can be induced by various cellular stresses, such as elevated intracellular reactive oxygen species (ROS) and nitric oxide (NO). To investigate the involvement of ROS in 6-OHDA-induced death of PC12 cells, the cells were treated with 250 μM 6-OHDA for 30 min, 1 h, 2 h and 3 h, followed by ROS measurement using the 2,7-dichlorofluorescein diacetate (DCF-DA) assay. DCF-DA assay showed that 6-OHDA significantly increased the levels of intracellular ROS in PC12 cells in a dose-dependent manner (Fig. 5A).

NO has been found to initiate both pro- and anti-apoptotic events in the brain (Choi *et al*., 2004). To test the involvement of NO in 6-OHDA-induced death of PC12 cells, cells were treated with 250 μM 6-OHDA for 30 min, 3 h, 5 h and 7 h, followed by measuring NO levels by NO assay. 6-OHDA markedly increased nitric oxide (NO) production in both a dose- and time-dependent manner (Fig. 5B).



	Concentrations (μM)				
	50	100	150	200	250
Cell survival of cells (% of control)	97.3 ± 2.2	87.0 ± 2.1 ***	76.0 ± 3.0 ***	69.1 ± 0.9 ***	56.0 ± 3.3 ***

Figure 2. Effect of 6-OHDA on PC12 cells viability. PC12 cells (1.0 × 105 cells/㎖) were treated with 250 μM 6-OHDA for 24 h at the indicated concentrations. The percentage of viable cells was estimated using the MTT assay. Results are mean ± SD (n=3). $^{***}p<0.005$ vs. control using student's t-test.

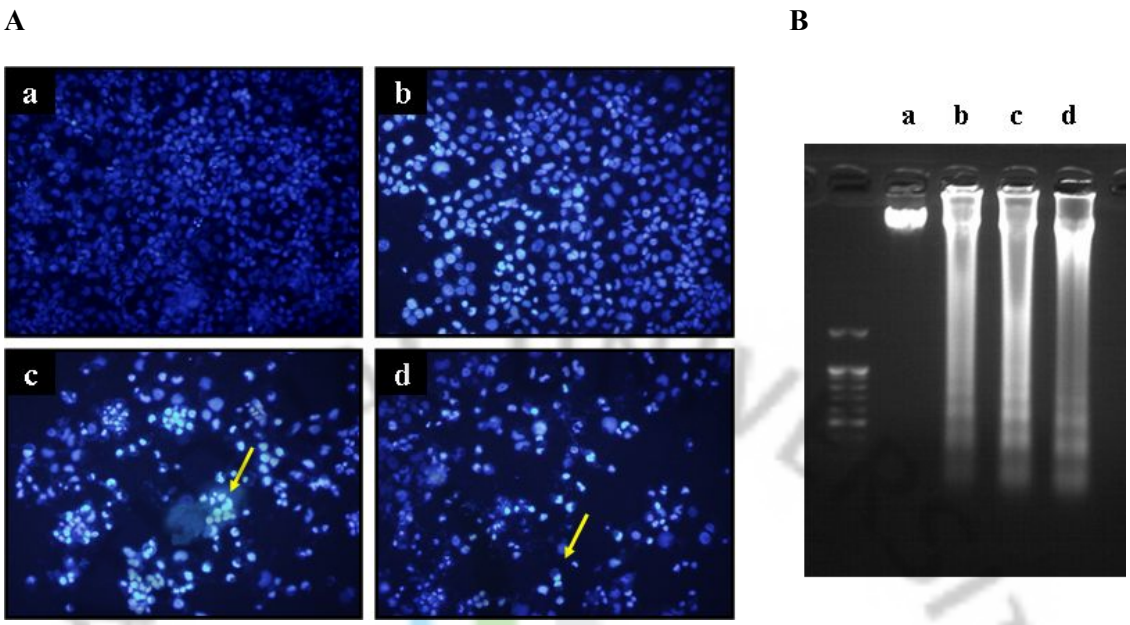


Figure 3. 6-OHDA-induced apoptosis in PC12 cells. (A) 6-OHDA-induced chromatin condensation. PC12 cells (1.0×10^5 cells/㎖) were plated on 24-well plates for 24 h, and the cells were treated with 250 μM 6-OHDA. Chromatin condensation was revealed by Hoechst 33342 staining; arrows indicate apoptotic cells. a; control, b; 250 μM 6-OHDA for 3 h, c; 250 μM 6-OHDA for 5 h, d; 250 μM 6-OHDA for 7 h. (B) 6-OHDA-induced DNA fragmentation. PC12 cells (1.0×10^5 cells/㎖) were treated with 250 μM 6-OHDA. a; control, b; 250 μM 6-OHDA for 3 h, c; 250 μM 6-OHDA for 5 h, d; 250 μM 6-OHDA for 7 h.

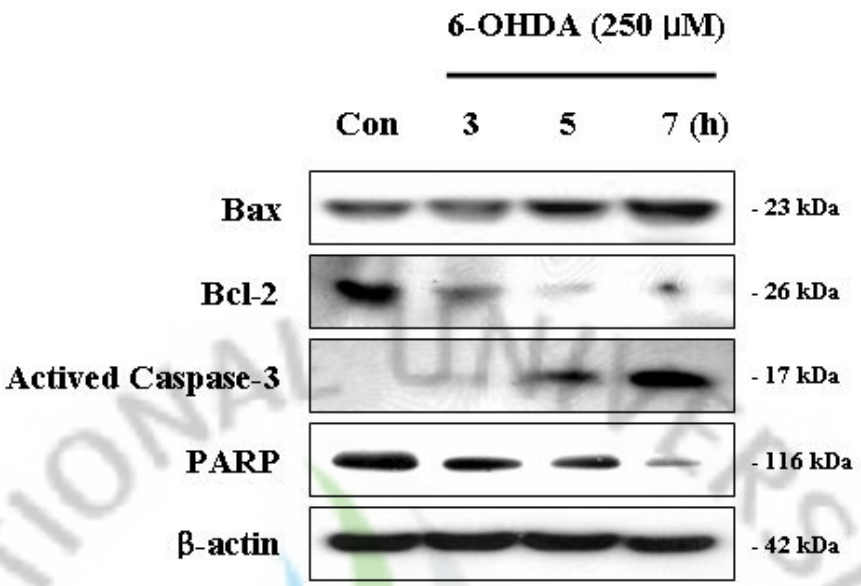
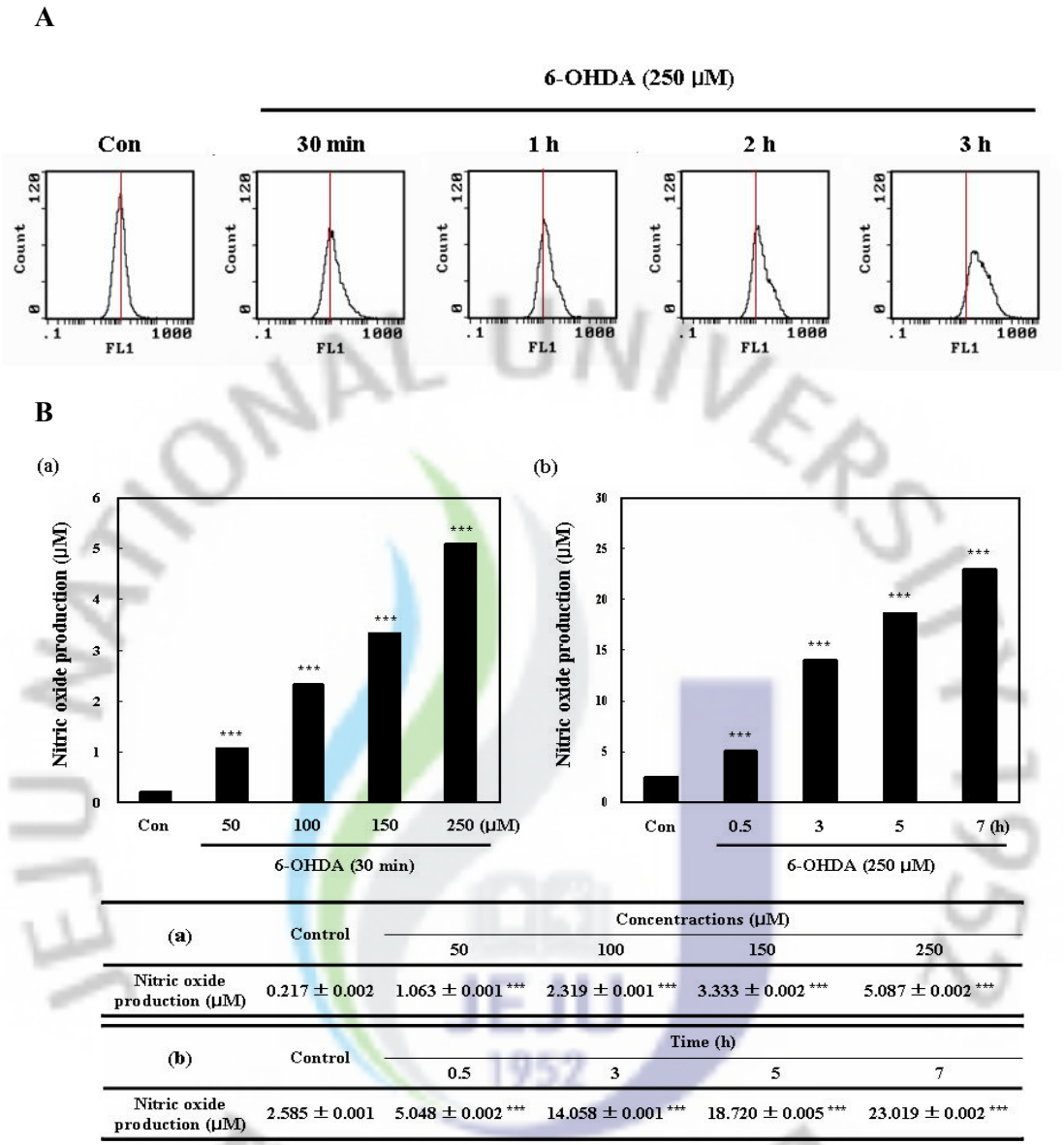


Figure 4. Induced levels of Bax, Bcl-2, caspase-3 and PARP in PC12 cells by 6-OHDA. PC12 cells (1.0×10^5 cells/㎖) were treated with 250 μM 6-OHDA for 3 h, 5 h and 7 h; Levels of Bax, Bcl-2, Caspase-3, PARP and β-actin protein were determined by western blotting. Molecular weight standards (kDa) are indicated to the right of the blot.



(a)	Control	Concentractions (μM)			
		50	100	150	250
Nitric oxide production (μM)	0.217 ± 0.002	1.063 ± 0.001 ***	2.319 ± 0.001 ***	3.333 ± 0.002 ***	5.087 ± 0.002 ***

(b)	Control	Time (h)			
		0.5	3	5	7
Nitric oxide production (μM)	2.585 ± 0.001	5.048 ± 0.002 ***	14.058 ± 0.001 ***	18.720 ± 0.005 ***	23.019 ± 0.002 ***

Figure 5. Generation of reactive oxygen species (ROS) and nitric oxide (NO) in PC12 cells by 6-OHDA. (A) PC12 cells (1.0×10^5 cells/㎖) were treated with 250 μM 6-OHDA for 30 min, 1 h, 2 h and 3 h; Reactive oxygen species (ROS) formation was measured using DCF-DA assay. (B) PC12 cells (1.0×10^5 cells/㎖) were treated with (a) 6-OHDA for 30 min at the indicated concentrations; (b) 250 μM 6-OHDA for 30 min, 1 h, 2 h and 3 h. Nitric oxide (NO) formation was measured using griess reagent. Results are mean ± SD (n=3). *** p<0.005 vs. control using student's t-test.

1.4. 6-OHDA-induced death of PC12 cells correlates with the degradation of MEF2D

Previous studies have shown expressions of MEF2 isoforms in the nucleus of cortical neurons (Gong *et al*., 2003). To determine if PC12 cells expressed endogenous MEF2D and if MEF2D was located in the nucleus, we performed western blots using specific MEF2D antibody. PC12 cells did express endogenous MEF2D and MEF2D was located in the nucleus (Fig. 6). We also investigated if 6-OHDA regulated MEF2D protein in PC12 cells. Exposure to 6-OHDA induced dose- and time-dependent decreases of MEF2D levels in PC12 cells (Fig 7A, B).

1.5. 6-OHDA-induced MEF2D downregulation is mediated by both Calpain and Cdk5 signaling

The Calpain and Cdk5-mediated pathways play essential roles in the downregulation of MEF2D (Gong *et al*., 2003; Smith *et al*., 2006). To determine if PD151746 or roscovitine protected PC12 cells from 6-OHDA-induced neuronal cell death, cell viability was estimated using the MTT assay after PC12 cells were pretreated with PD151746 or roscovitine in the presence of 6-OHDA. PD151746 treatment (81.5%) and roscovitine treatment (88.9%) prevented PC12 cells death (79.9%) induced by treatment with 250 μM 6-OHDA for 5 h (Fig. 8A). Next, to test for direct roles of Calpain and Cdk5-mediated pathways in MEF2D downregulation by 6-OHDA, Calpain and Cdk5 activities were inhibited using PD151746 and roscovitine, respectively. Pretreatment with either PD151746 or roscovitine blocked 6-OHDA-induced MEF2D downregulation (Fig. 8B).

1.6. Activation of MAP kinase pathway appears to be involved with PC12 cell death induced by 6-OHDA

To ascertain a possible relationship between 6-OHDA-induced PC12 cell death and MAP

kinases, we analyzed the phosphorylations of ERK1/2, JNK and p38 MAPK using western blot analyses. Phosphorylations of ERK1/2, JNK and p38 MAPK were markedly increased after 250 μM 6-OHDA treatment compared to the control (Fig. 9). Also, phosphorylations of ERK1/2 and JNK reached plateaus after 250 μM 6-OHDA treatment for 2 hr, and were maintained thereafter (Fig. 9).

1.7. 6-OHDA-induced death of PC12 cells involved in the inactivation of Akt pathway

The PI3K/Akt pathway plays an important role in neuronal survival (Wiedmann *et al*., 2005; Chong *et al*., 2005; Brunet *et al*., 2001). We assessed the phosphorylation of Akt using western blot analysis, and found decreased phosphorylation of Akt after 250 μM 6-OHDA treatment (Fig. 10).

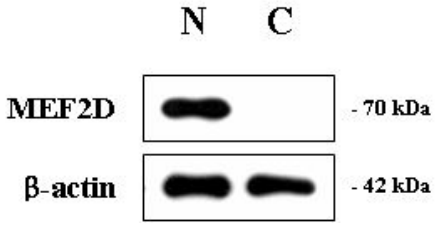
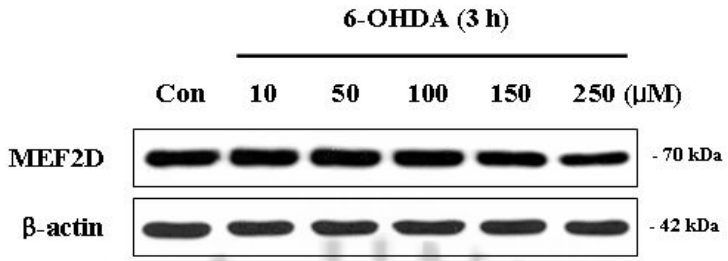


Figure 6. Expression of endogenous MEF2D in PC12 cells. PC12 cells (1.0×10^5 cells/㎖) were fractionated into cytoplasmic or nuclear extracts; The subcellular distribution of MEF2D was determined by western blotting. β-actin was used as a loading control after reprobing the same membrane.

A



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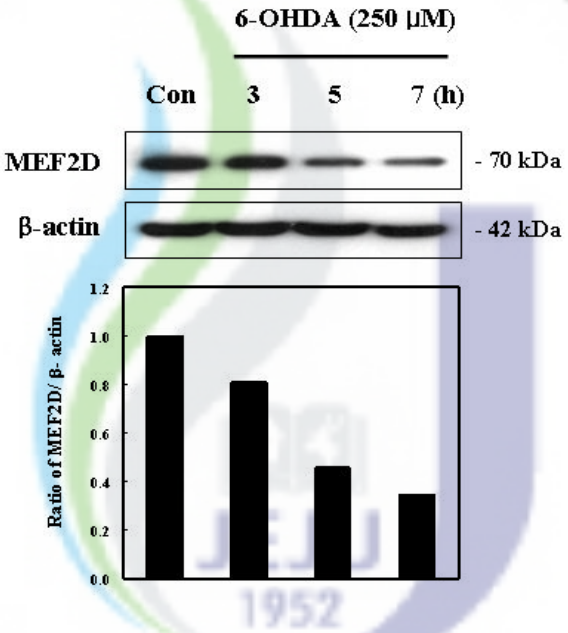
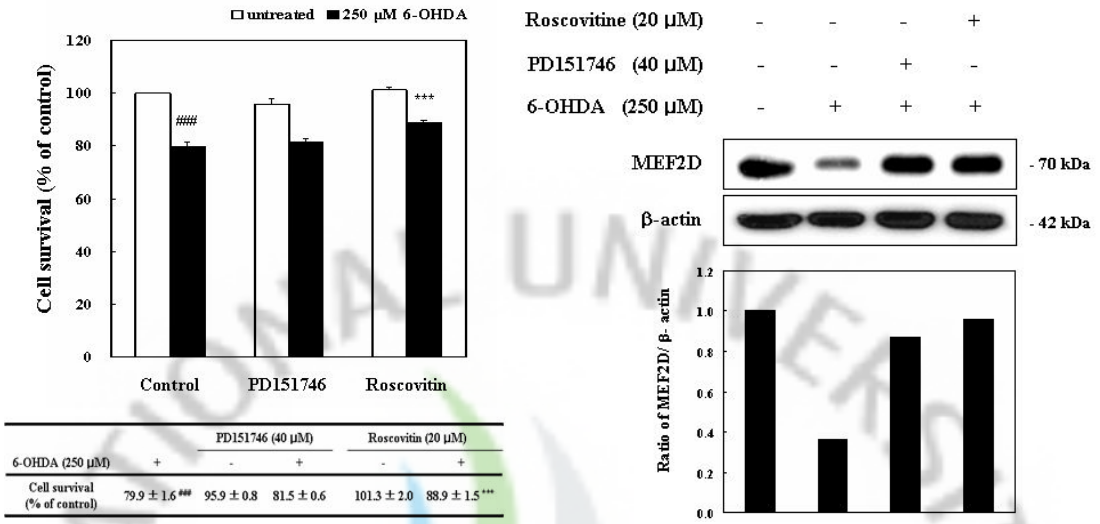


Figure 7. Downregulation of MEF2D in PC12 cells by 6-OHDA. 6-OHDA promoted degradation of MEF2D proteins in PC12 cells. (A) PC12 cells (1.0 × 105 cells/㎖) were treated with 6-OHDA for 3 h at the concentrations of 10, 50, 100, 150 and 250 μM. (B) PC12 cells (1.0 × 105 cells/㎖) were treated with 250 μM 6-OHDA for 3 h, 5 h and 7 h; Levels of MEF2D and β-actin protein were determined by western blotting after 6-OHDA treatment.

A **B**



		PD151746 (40 μM)		Roscovitin (20 μM)	
6-OHDA (250 μM)	+	-	+	-	+
Cell survival (% of control)	79.9 ± 1.6 ###	95.9 ± 0.8	81.5 ± 0.6	101.3 ± 2.0	88.9 ± 1.5 ***

Figure 8. Requirement for the Calpain/Cdk5 pathways on 6-OHDA-induced downregulation of MEF2D in PC12 cells. (A) PC12 cells (1.0×10^5 cells/㎖) were pretreated with PD151746 (Calpain inhibitor) or Roscovitine (Cdk5 inhibitor) and then treated with 250 μM 6-OHDA for 5 h. The percentage of viable cells was estimated using the MTT assay. Results are mean ± SD (n=3). $^{###}p<0.005$ vs. control, $^{***}p<0.005$ vs. 6-OHDA treatment control using student's t-test. (B) PC12 cells (1.0×10^5 cells/㎖) were pretreated with PD151746 (Calpain inhibitor) or roscovitine (Cdk5 inhibitor) and then treated with 250 μM 6-OHDA for 5 h; Level of MEF2D was determined by western blotting. β-actin was used as a loading control after reprobing the same membrane.

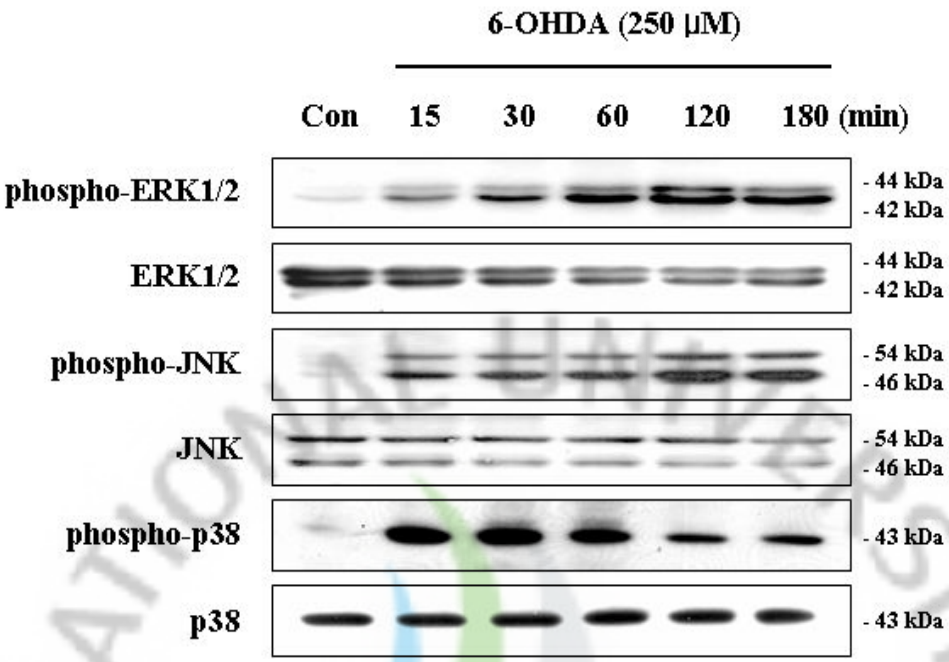


Figure 9. Induced levels of ERK 1/2, JNK and p38 MAPK in PC12 cells by 6-OHDA. PC12 cells (1.0×10^5 cells/㎖) were treated with 250 μM 6-OHDA for 15, 30, 60, 120 and 180 min; Expressions of the phosphorylated form of each MAPKs (ERK: Thr202/Tyr204; JNK: Thr183/Tyr185; p38: Thr180/Tyr182) and the corresponding total proteins were determined by western blots.

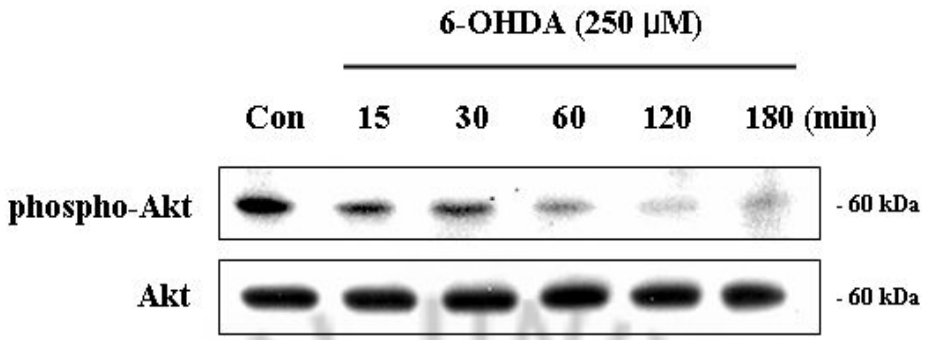


Figure 10. Induced levels of Akt in PC12 cells by 6-OHDA. PC12 cells (1.0×10^5 cells/㎖) were treated with 250 μM 6-OHDA for 15, 30, 60, 120 and 180 min; Expressions of the phosphorylated form of Akt (Ser473) and total Akt were determined by western blots.

1.8. Influence of Cdk5 on 6-OHDA-induced alterations of MAP kinase and PI3K/Akt signaling: Cdk5 and MAP kinase 'cross-talk'.

1.8.1. Cdk5 regulates the ERK1/2 pathway

The Ras-Raf-MEK-ERK pathway is stimulated by various growth factors and plays important roles in cell survival, differentiation and proliferation. This pathway interacts with other signals due to overlapping substrate specificities or shared regulatory sites (Pearson *et al*., 2001). To examine the effect of Cdk5 on 6-OHDA-induced activation of ERK 1/2, one of the MEK1 substrates, PC12 cells were treated with 6-OHDA for 2 h in the presence or absence of roscovitine (20 μM), an inhibitor of Cdk5. In the absence of roscovitine, 250 μM 6-OHDA treatment enhanced phosphorylation of ERK1/2 (Fig. 11A and C). Interestingly, when the cells were treated with 6-OHDA in the presence of roscovitine, ERK1/2 phosphorylation increased about 2.7-fold (Fig. 11A and C).

1.8.2. Cdk5 may affects the JNK and p38 MAPK pathways

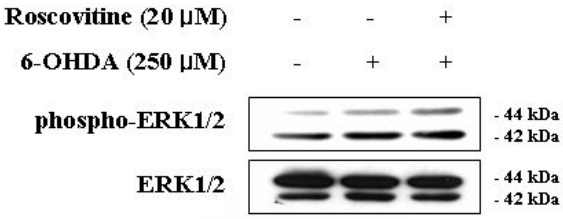
Cdk5 is also involved in regulating apoptotic signaling pathways. Cross-talk between Cdk5 and the c-Jun N-terminal kinase (JNK) pathway involved in neuronal apoptosis has been demonstrated (Zhang *et al*., 2002; Kyriakis *et al*., 1994). PC12 cells were treated with 6-OHDA for 2 h in the presence or absence of roscovitine (20 μM). 6-OHDA treatment (250 μM) increased the phosphorylation of JNK (Fig. 11B and C) and 20 μM roscovitine enhanced the 6-OHDA-induced increase of p-JNK (Fig. 11B and C). 6-OHDA also increased the phosphorylation of p38, while roscovitine downregulated 6-OHDA-induced p-p38 (Fig. 11B and C).

1.8.3. Cdk5 activity may not affects the PI3/Akt pathway in 6-OHDA-induced death of

PC12 cells

To investigate the effect of Cdk5 on the inactivation of the PI3/Akt pathway by 6-OHDA, PC12 cells were treated with 6-OHDA for 2 h in the presence or absence of roscovitine (20 μM). 6-OHDA treatment (250 μM) decreased the phosphorylation of Akt, and 20 μM roscovitine treatment did not affect this 6-OHDA-induced inactivation of Akt (Fig. 12).

A



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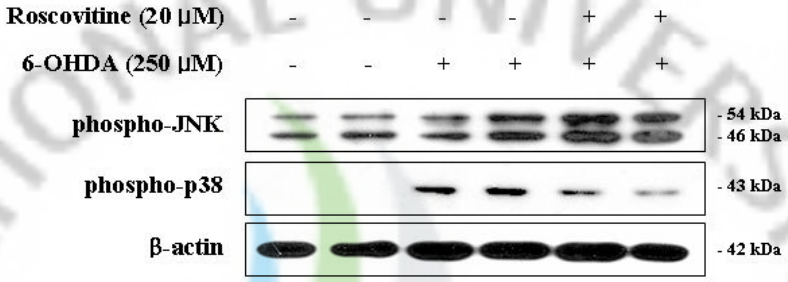


C

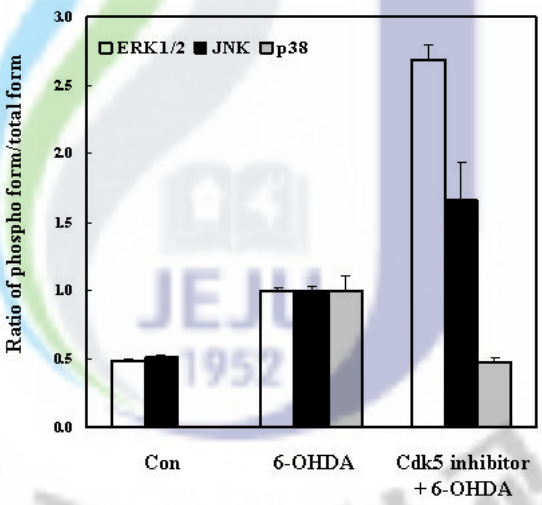


Figure 11. Effect of roscovitine on 6-OHDA-induced activation of MAPKs in PC12 cell.

PC12 cells (1.0×10^5 cells/㎖) were pretreated with roscovitine (Cdk5 inhibitor) and then treated with 250 μM 6-OHDA for 2 h; Levels of the phosphorylated form of each MAPK (ERK 1/2: Thr202/Tyr204; JNK: Thr183/Tyr185; p38: Thr180/Tyr182) and the corresponding total protein were determined by western blots.

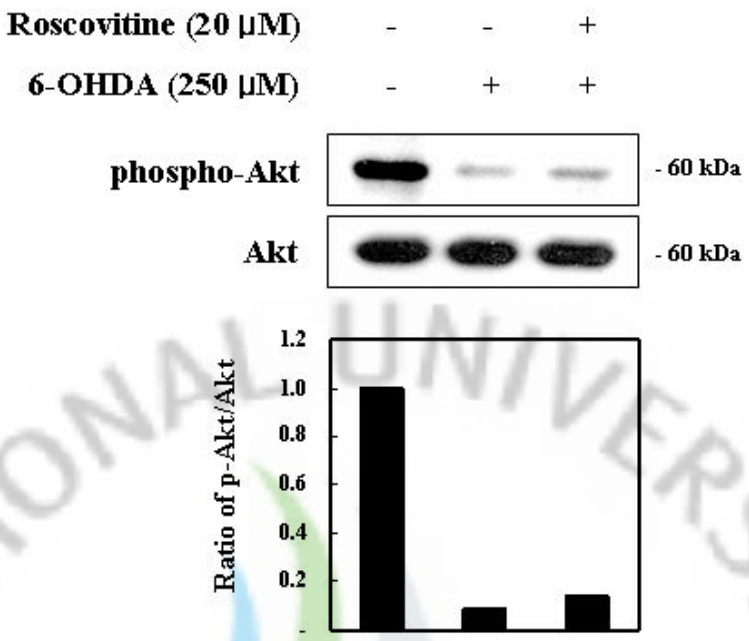


Figure 12. Effect of roscovitine on 6-OHDA-induced inactivation of Akt in PC12 cell. PC12 cells (1.0×10^5 cells/㎖) were pretreated with roscovitine (Cdk5 inhibitor) and then treated with 250 μM 6-OHDA for 2 hr; Levels of the phosphorylated form of Akt (Ser473) and the Akt were determined by western blots.

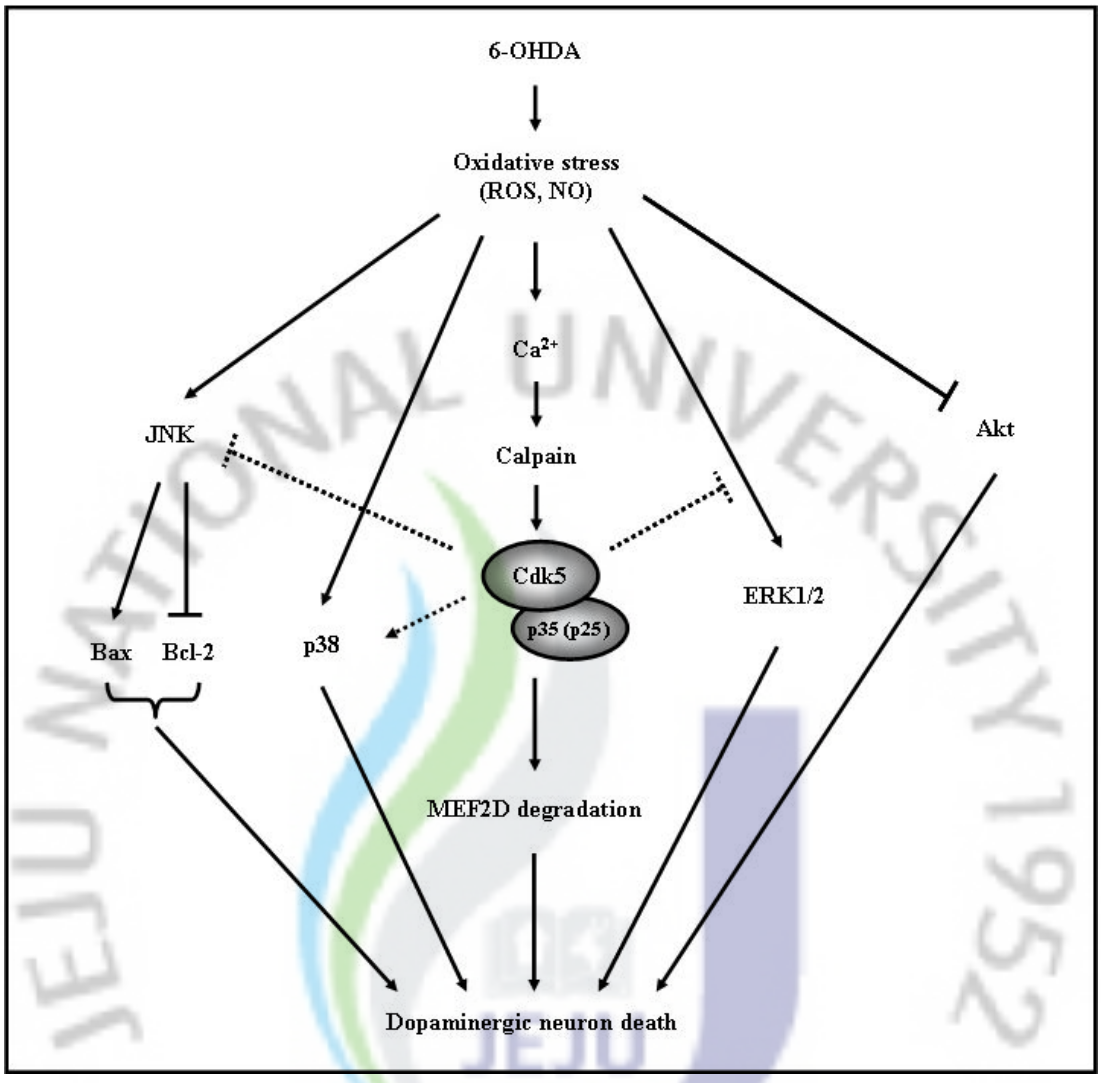


Figure 13. 6-OHDA-induced neuronal cell death in PC12 cells. Intracellular pathway involved with 6-OHDA-induced cell death in PC12 cells: 1) MEF2D downregulation, 2) inactivation of Akt, 3) activation of MAP Kinase; the three pathways are necessary for neuronal cell death, as blocking one pathway is sufficient to maintain cell viability.

2. Neuroprotective effect of *Carpinus tschonoskii* MAX on 6-hydroxydopamine-induced death of PC12 cells

2.1. *Carpinus tschonoskii* MAX protects PC12 cells against 6-OHDA-induced death

To determine if *Carpinus tschonoskii* MAX (*C. tschonoskii*) protected PC12 cells against 6-OHDA-induced neuronal cell death, the cell viability of *C. tschonoskii* 80% EtOH extract-treated PC12 cells was determined by MTT assay. *C. tschonoskii* extract prevented the PC12 cell death induced by 24 h treatment with 250 μM 6-OHDA in a dose-dependent manner (Fig. 14). PC12 cells were also pretreated with 50 or 100 ㎍/㎖ of EtOH extract and different solvent fractions of *C. tschonoskii* in the presence of 6-OHDA. There was markedly decreased C12 cell death following 6-OHDA exposure by pre-treatments with the EtOH extract, $CHCl_3$ fraction, EtOAc fraction and BuOH fraction (Fig. 15).

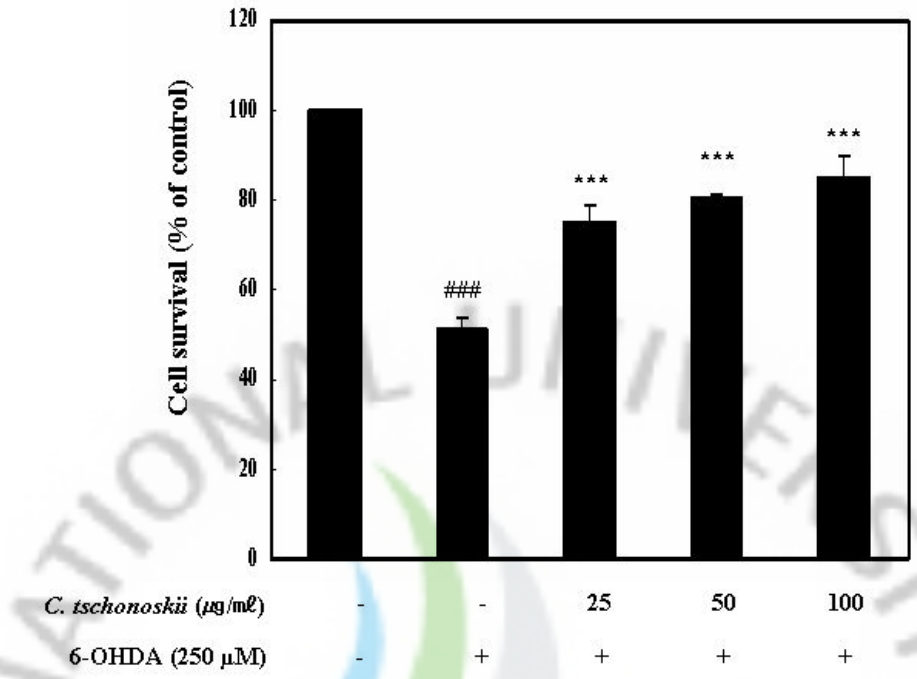
2.2. *Carpinus tschonoskii* MAX attenuates 6-OHDA-induced apoptosis in PC12 cells

The protective effect of *C. tschonoskii* against 6-OHDA-induced apoptosis was examined by measuring nuclear condensation using Hoechst 33342. Chromatin condensation was increased by treatment with 250 μM 6-OHDA for 7 h, while 100 ㎍/㎖ of *C. tschonoskii* extract significantly reduced 6-OHDA-induced apoptosis (Fig. 16A). Consistently, DNA fragmentation was increased by treatment with 250 μM 6-OHDA for 7 h, and 100 ㎍/㎖ of *C. tschonoskii* EtOH extract significantly reduced 6-OHDA-induced DNA fragmentation (Fig. 16B). We also studied any neuroprotective effects of *C. tschonoskii* on the activation of apoptotic signaling by 6-OHDA. PC12 cells were treated with 250 μM 6-OHDA for 7 h after 1, 10, 50, 100 ㎍/㎖ of *C. tschonoskii* EtOH extract pre-treatment. 6-OHDA increased the expression of Bax, while the expressions of Bcl-2, procaspase-3 and PARP were decreased. These effects were rescued by *C. tschonoskii* pretreatment in a dose-dependent manner (Fig.

17).

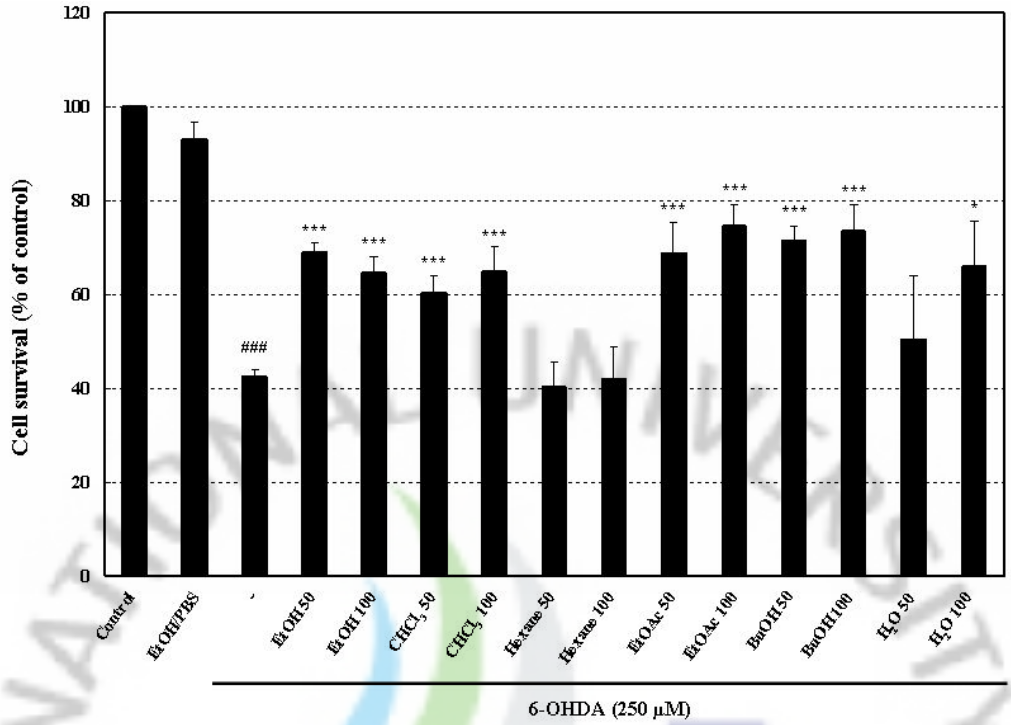
2.3. *Carpinus tschonoskii* MAX reduces 6-OHDA-induced generation of intracellular ROS and NO

6-OHDA promoted the formation of intracellular ROS and NO (Fig. 5A, B). Thus, we examined if *C. tschonoskii* inhibited the ROS and NO signals associated with 6-OHDA-induced cell death. PC12 cells were treated with 250 μM 6-OHDA for 30 min after pretreatment with *C. tschonoskii* EtOH extract, and then intracellular ROS was measured by 2,7-dichlorofluorescein diacetate (DCF-DA) assay. The *C. tschonoskii* extract significantly reduced the 6-OHDA-induced ROS generation, indicating that the *C. tschonoskii* extract attenuated the pro-oxidant effects of 6-OHDA (Fig. 18A). Next, we assessed the effects of *C. tschonoskii* on 6-OHDA-induced formation of NO. 6-OHDA significantly increased NO levels in both a dose- and time-dependent manner (Fig. 4B); while this was suppressed by *C. tschonoskii* extract (Fig. 18B).



	Control (6-OHDA)	Concentractions (μg/mℓ)		
		25	50	100
Cell survival of PC12 cells (% of control)	51.1 ± 3.0 ###	74.7 ± 4.3***	80.6 ± 0.8 ***	84.8 ± 5.3 ***

Figure 14. Neuroprotective effect of *Carpinus tschonoskii* MAX on 6-OHDA-induced death of PC12 cells. PC12 cells (1.0 × 105 cells/㎖) were pretreated with 25, 50 and 100 ㎍/㎖ of 80% EtOH extracts from *C. tschonoskii* prior to 250 μM 6-OHDA exposure and measured for viability by MTT assay for 24 h. Results are mean ± SD (n=3). ###p<0.005 vs. control, ***p<0.005 vs. 6-OHDA treatment group using student's t-test.



Concentrations (μg/mℓ)	Control (6-OHDA)	Cell survival of PC12 cells (% of control)					
		80% EtOH	$CHCl_3$	Hexane	EtOAc	BuOH	H_2O
50	42.6 ± 1.2 ###	69.1 ± 1.9 ***	60.3 ± 3.7 ***	40.2 ± 5.6	68.8 ± 6.7 ***	71.4 ± 3.2 ***	50.4 ± 13.4
100		64.6 ± 3.4 ***	64.9 ± 5.1 ***	42.1 ± 6.9	74.6 ± 4.6 ***	73.6 ± 5.6 ***	65.9 ± 9.6 *

Figure 15. Neuroprotective effect of EtOH extract or solvent fractions from *Carpinus tschonoskii* MAX on 6-OHDA-induced death of PC12 cells. PC12 cells (1.0 × 105 cells/㎖) were treated with 250 μM 6-OHDA after 50 and 100 ㎍/㎖ of 80% EtOH extract or solvent fractions from *C. tschonoskii* pretreatment and measured for viability by MTT assay for 24 h. Results are mean ± SD (n=3). $^{###}p<0.005$ vs. control, $^{*}p<0.05$ vs. 6-OHDA treatment group, $^{***}p<0.005$ vs. 6-OHDA treatment group using student's t-test.

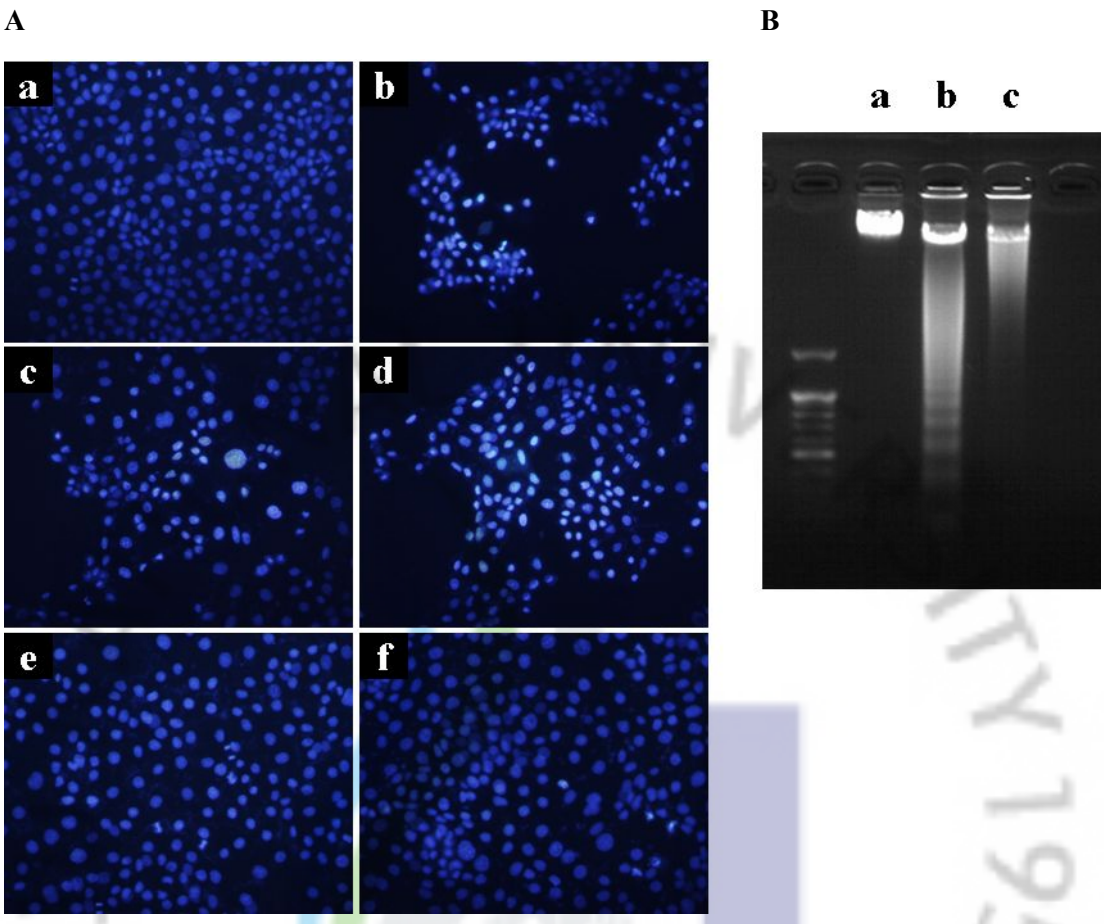


Figure 16. Neuroprotective effect of *Carpinus tschonoskii* MAX on the 6-OHDA-induced apoptosis in PC12 cells. (A) *C. tschonoskii* reduced 6-OHDA-induced chromatin condensation. PC12 cells (1.0×10^5 cells/㎖) were treated with a; control, b; 6-OHDA treatment for 7 h, c; 1 ㎍/㎖ of *C. tschonoskii* for 30 min prior to 6-OHDA treatment for 7 h, d; 10 ㎍/㎖ of *C. tschonoskii* for 30 min prior to 6-OHDA treatment for 7 h, e; 50 ㎍/㎖ of *C. tschonoskii* for 30 min prior to 6-OHDA treatment for 7 h, f; 100 ㎍/㎖ of *C. tschonoskii* for 30 min prior to 6-OHDA treatment for 7 h. (B) *C. tschonoskii* inhibited 6-OHDA-induced DNA fragmentation. PC12 cells (1.0×10^5 cells/㎖) were treated with a; control, b; 250 μM 6-OHDA for 7 h, c; 250 μM 6-OHDA treatment for 7 h after pretreatment of *C. tschonoskii* (100 ㎍/㎖).

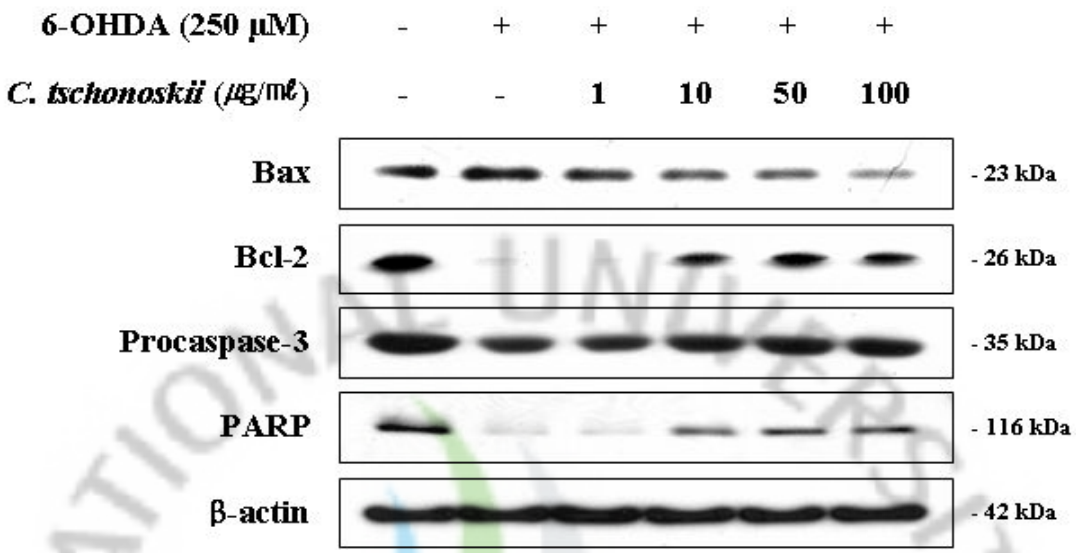
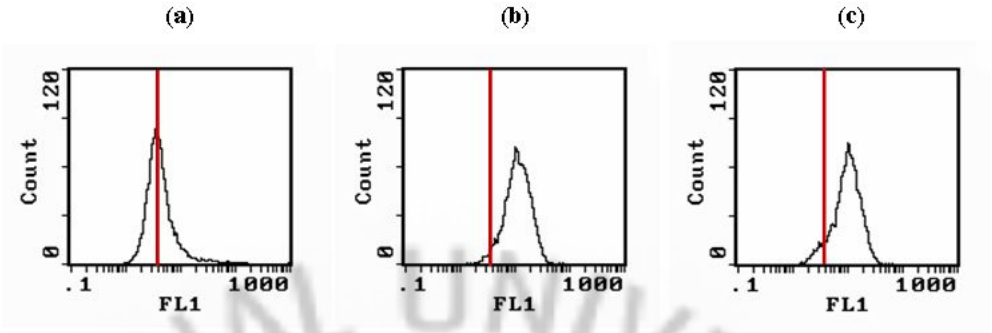


Figure 17. Neuroprotective effect of *Carpinus tschonoskii* MAX on 6-OHDA-induced apoptotic signaling in PC12 cells. PC12 cells (1.0×10^5 cells/mℓ) were treated with 250 μM 6-OHDA for 7 h after 1, 10, 50 and 100 μg/mℓ of *C. tschonoskii* pretreatment; Expressions of Bax, Bcl-2, proaspase-3, PARP and β-actin protein were determined by western blotting.

A



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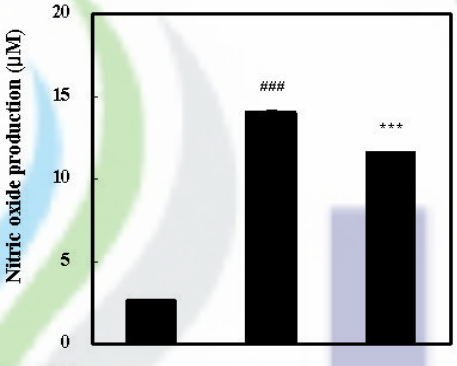

C. tschonoskii (100 μg/mℓ)	-	-	+
6-OHDA (250 μM)	-	+	+

	Control	6-OHDA (250 μM)	
		-	*C. tschonoskii* (100 μg/mℓ)
Nitric oxide production (μM)	2.585 ± 0.001	14.058 ± 0.001 ###	11.570± 0.003 ***

Figure 18. Neuroprotective effect of *Carpinus tschonoskii* MAX on the 6-OHDA-induced increases of ROS and NO in PC12 cells. (A) *C. tschonoskii* suppressed 6-OHDA-induced intracellular ROS. PC12 cells (1.0×10^5 cells/㎖) were pretreated with *C. tschonoskii* (100 ㎍/㎖) for 30 min prior to 6-OHDA treatment for 30 min. a; control, b; 250 μM 6-OHDA for 3 h, c; 250 μM 6-OHDA treatment for 3 h after pretreatment of *C. tschonoskii* (100 ㎍/㎖). (B) *C. tschonoskii* reduced 6-OHDA-induced production of NO. PC12 cells (1.0×10^5 cells/㎖) were pretreated with *C. tschonoskii* (100 ㎍/㎖) for 30 min prior to 6-OHDA treatment for 3 h. Results are mean ± SD (n=3). $^{###}p<0.005$ vs. control, $^{***}p<0.005$ vs. 6-OHDA treatment group using student's t-test.

2.4. *Carpinus tschonoskii* MAX ameliorates 6-OHDA-induced downregulation of MEF2D

We next assessed if *C. tschonoskii* could ameliorate the decreases of MEF2D levels induced by 6-OHDA. PC12 cells were pretreated with *C. tschonoskii* at final concentrations of 1, 10, 50, 100 ㎍/㎖ for 30 min, and then treated with 250 μM 6-OHDA for 7 h. *C. tschonoskii* treatment dose-dependently increased MEF2D expression (Fig. 19).

2.5. *Carpinus tschonoskii* MAX affects 6-OHDA-induced activation of ERK1/2 and JNK

To determine if there were any effects of *C. tschonoskii* on the 6-OHDA-induced activation of MAPK pathways, PC12 cells were pretreated with *C. tschonoskii* at final concentrations of 1, 10, 50, 100 ㎍/㎖ for 30 min, and then treated with 250 μM 6-OHDA for 2 h. Treatment of PC12 cells with 6-OHDA increased the phosphorylations of ERK 1/2, JNK and p38 (Fig. 9), while treatment with *C. tschonoskii* EtOH extract decreased the phosphorylations of ERK 1/2 and JNK in a dose-dependent manner (Fig. 20). However, *C. tschonoskii* EtOH extract did not affect 6-OHDA-induced phosphorylation of p38 (Fig. 20).

2.6. *Carpinus tschonoskii* MAX recovers 6-OHDA-induced inactivation of Akt

We investigated if *C. tschonoskii* could ameliorate inactivation of Akt by 6-OHDA. PC12 cells were pretreated with *C. tschonoskii* at final concentrations of 1, 10, 50, 100 ㎍/㎖ for 30 min, and then treated with 250 μM 6-OHDA for 2 h. Treatment of PC12 cells with 6-OHDA decreased the phosphorylation of Akt (Fig. 10), while the treatment with *C. tschonoskii* EtOH extract increased the phosphorylation of Akt in a dose-dependent manner (Fig. 21).

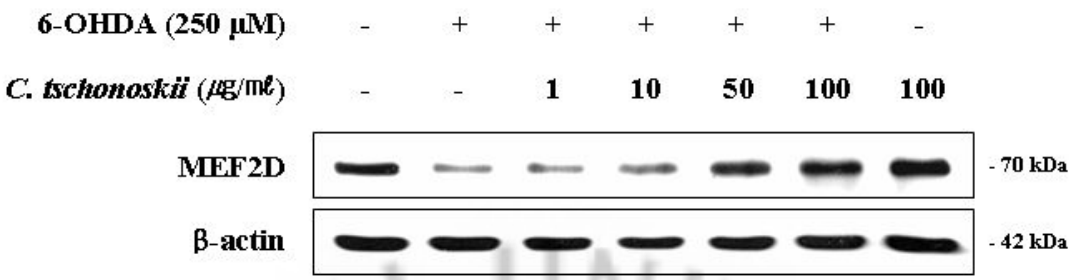

Figure 19. Neuroprotective effect of *Carpinus tschonoskii* MAX on 6-OHDA-induced downregulation of MEF2D in PC12 cells. PC12 cells (1.0×10^5 cells/㎖) were treated with 250 μM 6-OHDA for 7 h after 1, 10, 50 and 100 μg/㎖ of *C. tschonoskii* pretreatment; Level of MEF2D was determined by western blotting. β-actin was used as a loading control after reprobing the same membrane.

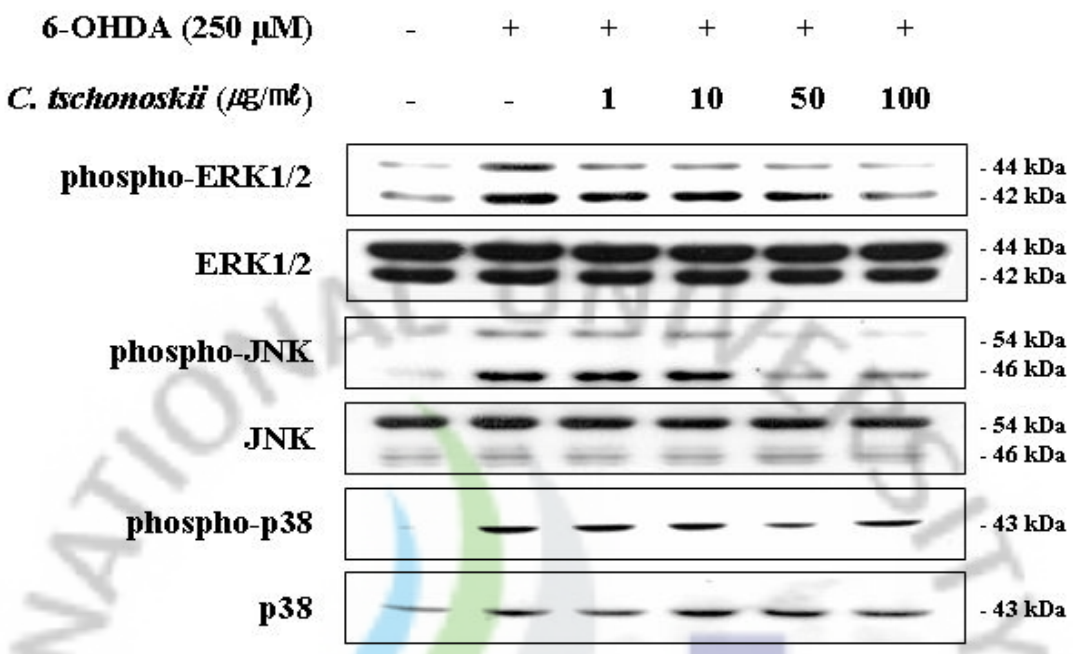

Figure 20. Neuroprotective effect of *Carpinus tschonoskii* MAX on 6-OHDA-induced activation of MAP kinase in PC12 cells. PC12 cells (1.0×10^5 cells/mℓ) were treated with 250 μM 6-OHDA for 2 h after 1, 10, 50 and 100 μg/mℓ of *C. tschonoskii* pretreatment; Levels of the phosphorylated form of each MAPK (ERK: Thr202/Tyr204; JNK: Thr183/Tyr185; p38: Thr180/Tyr182) and the corresponding total protein were determined by western blots.

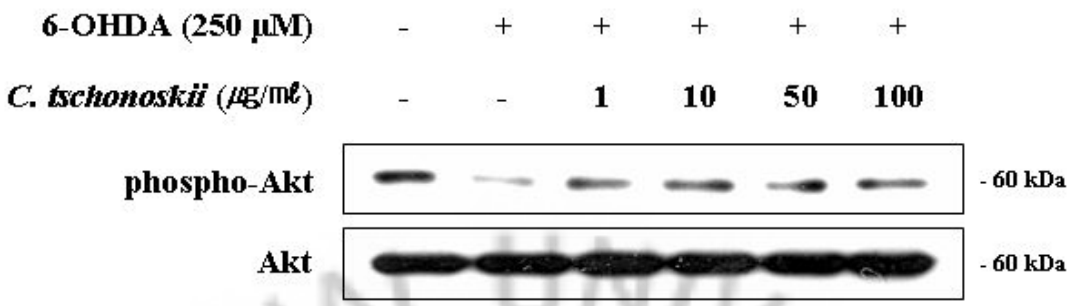

Figure 21. Neuroprotective effect of *Carpinus tschonoskii* MAX on 6-OHDA-induced inactivation of Akt in PC12 cells. PC12 cells (1.0×10^5 cells/㎖) were treated with 250 μM 6-OHDA for 2 h after 1, 10, 50 and 100 μg/㎖ of *C. tschonoskii* pretreatment; Levels of the phosphorylated form of Akt (Ser473) and total Akt were determined by western blots.

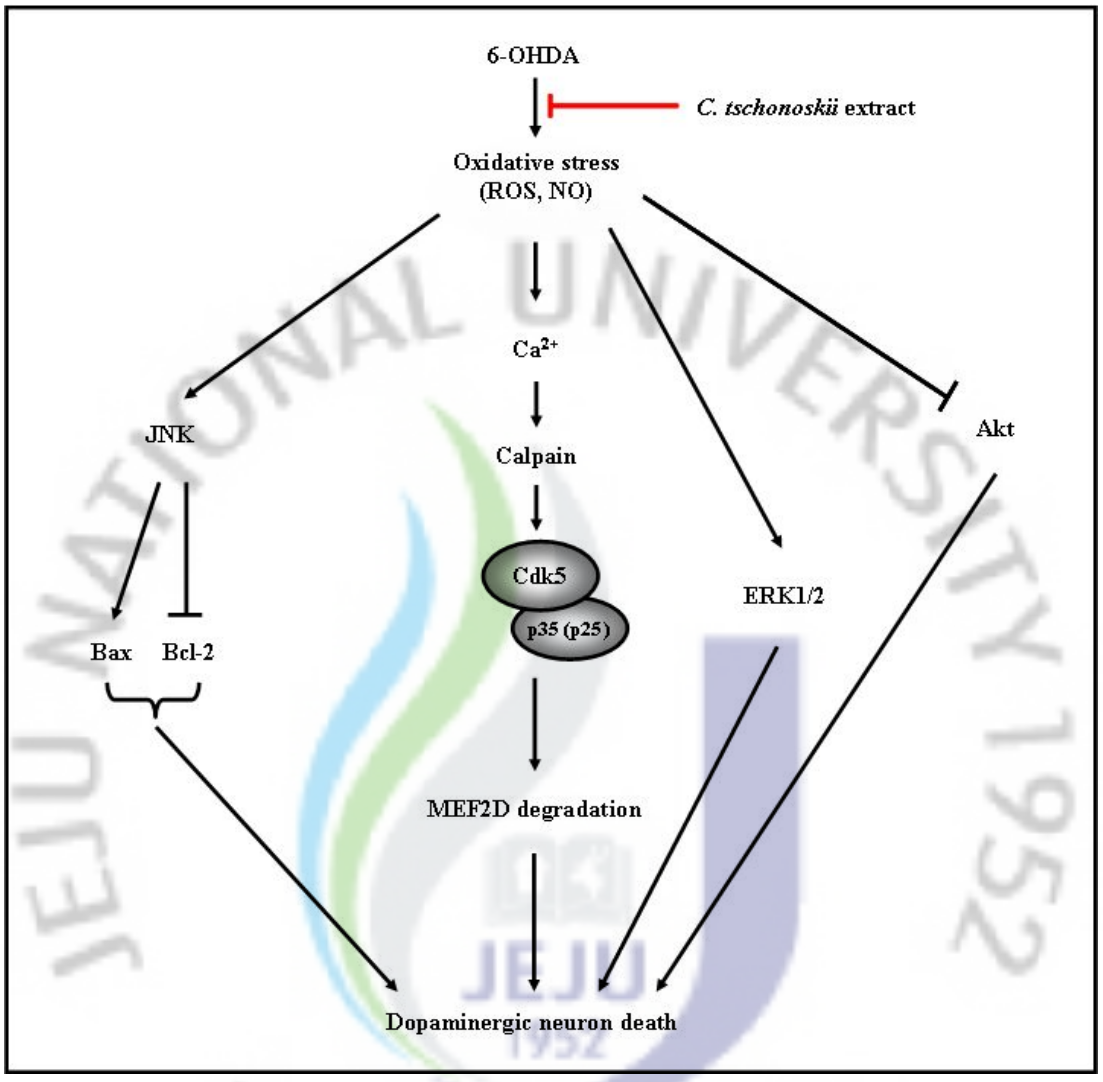

Figure 22. Neuroprotective effect of *Carpinus tschonoskii* MAX on 6-OHDA-induced neuronal cell death of PC12 cells. *C. tschonoskii* extract protects against PC12 cell death by 6-OHDA via suppressing oxidative stresses, such as ROS and NO. As a result, *C. tschonoskii* treated cells show attenuated downregulation of MEF2D, inactivation of Akt, activation of ERK1/2 and JNK and apoptotic signaling induced by 6-OHDA in PC12 cells.

Ⅳ. DISCUSSION

To the best of our knowledge, this study is the first to demonstrate that 6-OHDA-induced death of PC12 cells might be involved in MEF2D degradation, a critical transcription factor for neuronal survival. We demonstrated that the 6-OHDA-induced down regulation of MEF2D was mediated by both Calpain and Cdk5. We also found that the Cdk5/p35 pathway cross-talks with MAP kinase, which may balance the activities leading to either neuronal survival or apoptosis. Together with our results reported in a companion paper, we confirmed a neuroprotective effect of *C. tschonoskii* on 6-OHDA-induced death of PC12 cells.

Our results show that treatment with 6-OHDA caused PC12 cells death (Fig. 2) involving the generation of oxidative stress, including reactive oxygen species (ROS) (Fig. 5A) and nitric oxide (NO) (Fig. 5B). We also observed that 6-OHDA induced apoptosis. This was shown by nuclear chromatin condensation (Fig. 3A), DNA fragmentation (Fig. 3B), increased Bax expression (Fig. 4), decreased Bcl-2 expression (Fig. 4), activation of caspase-3 (Fig. 4) and the cleavage of poly-(ADP-ribose) polymerase (PARP) (Fig. 4). These results indicate that 6-OHDA triggers PC12 cells apoptosis.

The death of midbrain dopaminergic neurons in Parkinson's disease (PD) may involve altered activities of survival signaling factors, including myocyte enhancer factor 2 D (MEF2D) (Mao *et al*., 1999), MAP kinase pathways (Veeranna *et al*., 2000) or Akt (Brunet *et al*., 2001). Previous studies showed that MEF2D plays an important role in neuronal survival (Mao *et al*., 1999; Mao and Wiedmann, 1999; Okamoto *et al*., 2000; Li *et al*., 2001; Gong *et al*., 2003; Liu *et al*., 2003). These papers showed that degradation of MEF2D, a critical transcription factor for neuronal survival, was involved in death of cortical neurons due to MPP^+ (Gong *et al*., 2003; Tang *et al*., 2005).

To investigate if transcriptional activity of MEF2D was regulated by 6-OHDA in PC12 cells, we first investigated if PC12 cells expressed endogenous MEF2D. We found that MEF2D was expressed in the nuclei of PC12 cells (Fig. 6) and that 6-OHDA treatment caused decreases of MEF2D in both dose- and time-dependent manners (Fig 7A, B). This suggests that 6-OHDA-induced PC12 cells death might involve MEF2D down-regulation.

Recent studies have shown that the Calpain-mediated p35/Cdk5 pathway increased the phosphorylation of MEF2D (Gong *et al*., 2003; Tang *et al*., 2005; Smith *et al*., 2006). Cdk5-mediated phosphorylation of MEF2Dat the Serine 444 inactivating site results in a loss of dopaminergic neurons due to MPTP. (Tang *et al*., 2005; Smith *et al*., 2006). Using the Calpain inhibitor PD151746 and the Cdk5 inhibitor roscovitine, MEF2D that was down-regulated by 6-OHDA showed recovery to untreated levels (Fig. 8). These results demonstrate that the Calpain/Cdk5 pathway could lead to the inhibition of MEF2D in 6-OHDA-induced death of PC12 cells.

Mitogen-activated protein kinases (MAPK) and the PI3K/Akt pathway play a central role in cell death and survival (Jezabel *et al*., 2008; Chong *et al*., 2005; Sevgi *et al*., 2004; Veeranna *et al*., 2000; Brunet *et al*., 2001). After exposure to 6-OHDA, we found that phosphorylations of MAPKs, including ERK1/2, JNK and p38 MAPK, were markedly increased (Fig. 9). In contrast, the phosphorylation of Akt was decreased by 6-OHDA treatment (Fig. 10). These results suggest that MAP Kinase activation and Akt inactivation by 6-OHDA appear to play roles in PC12 cell death. These results describe an intracellular pathway by which MEF2D downregulation, MAPK activation and Akt inactivation converge for apoptotic signaling to induce neuronal cell death (Fig. 13).

Cdk5 kinase activity is also involved in ‘cross-talk’ and regulation of other signal pathways, including MAP kinase (Fig. 1). Both Cdk5 and MAP kinase pathways play critical roles during the development of the nervous system. Previous studies reported that Cdk5-induced

MEK1 phosphorylation at Thr286 suppressed its transcriptional activity both *in vitro* and *in vivo* (Sharma *et al*., 2002). Cdk5-induced inactivation of MEK1 promoted a feedback down-regulation of the MAP kinase signal cascade (Harada *et al*., 2001; Sharma *et al*., 2002; Zheng *et al*., 2007). We determined if Cdk5 affected MAP kinase pathway in 6-OHDA-treated PC12 cells using roscovitine.

When PC12 cells were treated with 6-OHDA in the presence of roscovitine, there was an increase in phosphorylation of ERK1/2 (Fig. 11A and C). These results are consistent with the report of Sharma (2002) showing that Cdk5/p35 inhibits MEK1 activity and terminates the ERK1/2 activation response in NGF-stimulated PC12 cells. This suggests that Cdk5/p35 signaling cross-talks with the Ras-Raf-MEK1-ERK1/2 pathway for neuronal survival.

Cdk5 also inhibits JNK in HEK293T cells after UV irradiation (Zhang *et al*., 2002). Cdk5 directly phosphorylates JNK (Thr93 for rat JNK1/2, Thr131 for rat JNK3) to inhibit its kinase activity (Kyriakis *et al*., 1994). Previous studies also showed that p35/Cdk5 kinase associates with Rac, upstream of JNK and p38 signaling, in a GTP-dependent manner (Nikolic *et al*., 1998). We investigated the effects of Cdk5 on activation of JNK and p38 by 6-OHDA. In the presence of roscovitine, there was increased phosphorylation of JNK (Fig. 11B and C). This is consistent with other experiments showing that Cdk5 negatively regulated JNK in HEK293T cells after UV irradiation (Zhang *et al*., 2002). These studies showed that Cdk5 plays a role in JNK signaling by negatively regulating its activity during neuronal apoptosis and that Cdk5 activity may contribute to neuronal survival. We also investigated if Cdk5 affected the p38 MAPK pathway. As a result, roscovitine decreased phosphorylation of p38 by 6-OHDA (Fig. 11B and C). These experiments support the idea that Cdk5 promotes the p38 MAPK pathway in 6-OHDA-induced neuronal apoptosis. However, Cdk5 activity did not affect 6-OHDA-induced inactivation of Akt in PC12 cells (Fig. 12). Collectively, these findings indicate that maintenance of the appropriate balance

between cdk5 and MAP kinase activity is essential for development of neuronal cells.

We demonstrated that *C. tschonoskii* EtOH extract significantly prevented PC12 cell death that was induced by treatment with 6-OHDA (Fig. 14). We also showed that different fractions of *C. tschonoskii* EtOH extract markedly decreased PC12 cell death by 6-OHDA treatment. These included the $CHCl_3$ fraction, EtOAc fraction and BuOH fraction (Fig. 15). We also studied the neuroprotective effects of *C. tschonoskii* on apoptosis induction by 6-OHDA. Hoechst 33342 staining and DNA fragmentation were increased by 6-OHDA treatment in PC12 cells, but *C. tschonoskii* EtOH extract significantly reduced these effects (Fig. 16A and B). These results suggest that *C. tschonoskii* EtOH extract inhibited 6-OHDA-induced apoptosis of PC12 cells. In addition, *C. tschonoskii* EtOH rescued the expression of Bax, whereas the expressions of Bcl-2, procaspase-3 and PARP were increased (Fig. 17). These results suggest that *C. tschonoskii* pre-treatment shifted the balance between a pro-apoptotic regulator and anti-apoptotic regulators towards cell survival through apoptotic signaling pathways. 6-OHDA significantly increased the levels of intracellular ROS and NO in PC12 cells, while *C. tschonoskii* EtOH extract was able to attenuate these increases (Fig. 18A and B).

The MEF2D transcription factors play critical roles in neuronal survival and apoptosis (Black and Olson, 1998; Mao *et al*., 1999; Mao and Wiedmann, 1999; Okamoto *et al*., 2000; Li *et al*., 2001; Gong *et al*., 2003; Liu *et al*., 2003). We observed that *C. tschonoskii* EtOH extract ameliorated MEF2D downregulation by 6-OHDA (Fig. 19). These results suggest that *C. tschonoskii* protects PC12 cells from 6-OHDA-induced death by enhancing the expression of MEF2D, and that MEF2D transcriptional activity is involved in neuronal cell survival.

We also investigated if *C. tschonoskii* attenuated the activation of MAPK pathway induced by 6-OHDA. The *C. tschonoskii* EtOH extract decreased the activation of ERK 1/2 and JNK,

while phosphorylation of p38 was not affected (Fig. 20). Thus, the neuroprotective effect of *C. tschonoskii* EtOH extract might arise from inhibiting the activation of ERK 1/2 and JNK pathways, but not by inhibition of the p38 pathway. Also, *C. tschonoskii* EtOH extract did increase Akt phosphorylation (Fig. 21). This suggests that *C. tschonoskii* protected against PC12 cell death by enhancing the activation of Akt.

In summary, 6-OHDA induces PC12 cell death by increasing oxidative stress (ROS and NO). This results in the following effects that relate to apoptotic signaling: Calpain/Cdk5-mediated MEF2D downregulation; activation of MAPK; inactivation of Akt. *C. tschonoskii* attenuates dopaminergic neuronal cell death induced by 6-OHDA by inhibiting the generation of ROS and NO. As a result, the following neuroprotective effects are observed: upregulation of MEF2D; inactivation of ERK1/2 and JNK pathways; activation of Akt pathway. The net result is a reduction of apoptotic signaling (Fig. 22). Consequently, in order to develop new strategies for treating Parkinson's disease, it may be valuable to establish the detailed functional roles of *C. tschonoskii* during 6-OHDA-induced PC12 cell death.

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Ⅵ. Abstract in Korean

파킨슨병에서 도파민성 신경세포의 소실과 관련된 생화학적 기전은 아직도 명확하게 밝혀져 있지 않다. 최근 연구된 바에 의하면, MPTP 모델에서 Calpain과 Cdk5에 의해 neuronal survival에 중요한 transcription factor인 MEF2D의 과인산화가 신경세포의 사멸과 관련되었을 것이라는 보고가 있었다. 그러나 6-hydroxydopamine (6-OHDA)에 의한 PC12 세포의 사멸이 Calpain과 Cdk5에 의해 매개된 MEF2D의 감소와 관련되어 있는지에 대한 연구 보고는 아직까지 없으며, 이를 규명하는 것은 파킨슨병의 도파민성 신경세포의 소실 기전을 이해하는데 큰 기여를 할 것으로 사료된다.

이에 본 연구에서는 PC12 세포에서 6-OHDA이 농도 및 시간 의존적으로 MEF2D를 확연히 감소시키는 것을 확인하였다. 또한, 우리는 6-OHDA에 의해 감소된 MEF2D가 Calpain 억제제인 PD151746과 Cdk5 억제제인 Roscovitine에 의해 각각 증가되는 것을 확인하였다. 이것은 6-OHDA에 의해 감소된 MEF2D는 Calpain과 Cdk5의 활성 모두에 의존적임을 확인할 수 있었다. 한편으로, 세포의 죽음과 생존에서 중요한 역할을 수행하는 MAP kinase와 Akt의 인산화가 6-OHDA에 의해 어떻게 변하는지를 분석하였다. PC12 cell에 6-OHDA를 처리하였을 때, ERK1/2, JNK 및 p38의 인산화가 시간 의존적으로 증가된 반면, Akt의 인산화는 시간 의존적으로 감소되었다. 이러한 결과들로 미루어 보아, MEF2D downregulation, MAP Kinase의 활성화 및 Akt의 비활성화가 6-OHDA에 의한 PC12 세포 죽음에서 중요하게 작용한다고 사료될 수 있었다. 또한 Cdk5와 MAP kinase 사이의 관련성을 확인해 본 결과, Cdk5 억제제인 Roscovitine은 ERK1/2와 JNK의 인산화를 증가시키는 반면, p38의 인산화는 감소시켰다. 이 결과는 Cdk5와 MAP kinase 신호전달 사이가 관련되어 있음을 시사하고, 두 신호전달 사이의

관련성은 신경세포의 생존과 죽음을 결정하는데 중요한 역할을 할 것으로 사료된다. 우리는 또한 6-OHDA에 의한 PC12세포의 죽음에서 항산화효과가 있다고 알려진 개서어나무 (*Carpinus tschonoskii* MAX)의 신경보호효과를 조사하였다. 개서어나무는 6-OHDA에 의해 유도된 PC12 세포의 죽음을 다음과 같은 메커니즘을 통해 약화시켰다: 1) 증가된 산화적 스트레스 (ROS와 NO)를 억제시켰다; 2) 감소된 MEF2D를 회복시켰다; 3) 활성화된 ERK1/2와 JNK 신호전달을 감소시켰다; 4) 비활성화된 Akt의 인산화를 증가시켰다; 5) Bax의 발현을 감소시키고, Bcl-2, procaspase-3와 PARP의 발현은 증가시켰다. 종합하면, 개서어나무 추출물은 6-OHDA에 의한 PC12세포의 죽음을 예방하고, 이러한 결과는 개서어나무가 파킨슨병의 치료전략으로 이용 가능함을 제시한다.

주요어: 파킨슨병, 6-hydroxydopamine, PC12 세포, MEF2D, Cdk5, MAP kinase, PI3/Akt 경로, 개서어나무

감사의 글

나는 성급했고, 반성하지 않았으며, 원망만 하였다. 그래서 난 언제나 그 자리였다.

항상 제자리에 머물러 있던 제가 이 논문을 작성하기까지 많은 도움과 격려를 주신 분들께 이 글을 빌어 감사의 마음을 전합니다.

먼저 **강희경 교수님**... 너무나 부족한 저였기에 '교수님께 폐가 되진 않을까' 죄인 같기만 했습니다. 하지만 결과가 나오기까지 믿고 기다려주시면서 해보고 싶은 실험은 아끼지 않고 지원해주신 점... 감사 드립니다. 또한 옆집 언니같이 상큼하신 **유은숙 교수님**... 지금 옆에 계시지 않지만 늘 따뜻하고 감사했습니다. 또한 대학원 생활 동안 논문에 대한 조언을 아끼지 않고 세심하게 지도해주신 **박덕배 교수님**, **강지훈 교수님**께 감사 드리며, 마주칠 때마다 관심과 격려를 주신 **현진원 교수님**, **강현욱 교수님 이영기 교수님**, **홍성철 교수님, 이근화 교수님, 정영배 교수님, 배종면 교수님, 김수영 교수님**을 비롯한 의과대학 모든 교수님들께도 감사의 마음을 전합니다. 대학원세미나 및 저널클럽 수업으로 없는 시간 쪼개면서 저 뿐만 아니고 다른 대학원생들 모두의 발표력을 업그레이드(^^)시켜주신 **조문제 교수님**, **고영상 교수님**께 다시 한번 감사드리고, 같은 신경 연구한다고 반가워하신 **은수용 교수님**... 아무나 안보여 준다는 해마분리실험을 차분히 가르쳐주시며, rat 머리를 작두로 자르시는 모습이 떠오르네요~^^ 그 따뜻한 마음 충분히 느낄 수 있었습니다. 감사합니다.

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퍼머가어울리는강순언니2 **정일**오빠, 있는집자식 **재희**언니, 멍키라총이두려운부양 **혜진**이에게 감사의 마음을 전합니다. 그리고 정말 많은 조언을 아끼지 않은 유부남2 **상철**오빠, 감사하고 결혼 축하드려요~ 선배들 덕에 무사히 졸업합니다. 논문을 쓰는 내내 큰 힘이 되어준 붕어같이귀여운 **영미**언니, 일년에한번화장하는 **희경**언니, 엔화올라서좋아하는유부녀1 **정은**언니, 남을칭찬할줄아는 **미경**언니, 실험이가장쉬웠어요~ **경아**언니, 든든한지원군같은 **지은**언니, 도도한 **장예**샘, 노는곳엔반드시있는 **진영**이, 더살쪄야되는 **윤지현**샘, 젠틀한 **수길**샘, 동안 **지강**샘, 동생같은 **연희**, 날씬쟁이 **금희**... 늘 고마웠습니다. 또한 우리 사랑하는 졸업동기들 유부녀2 **보연**, 소년체전박군 **선순**, 양양A **은진**, 뽄쟁이 **은희**, 유쾌상쾌하신 **윤영**샘 ... 모두 고생했고, 많은 도움줘서 고마워요~

참다운 벗은 좋은 때는 초대해야만 나타나고, 어려울 때는 부르지 않아도 나타난다.

늘 내 옆을 지켜준 B・F... 애교쟁이 **지뇽이**, 나쁜뇨자 **현미**, 하나2세탄생축하해~ **하나**... 나는 너희들에게 진정한 친구되지 못하는데, 니들은 나에게 진정한 친구가 되어주는구나.. 니들이 있어서 정말 큰 힘이 되었다. 그리고 사랑하는 공일학번 동기들과 후배들.. 연락 잘 못하지만, 간간히라도 날 잊지 않고 연락해줘서 고맙고, 우영멤버인 **마료**언니, **꼼쓰**언니, **싸서코**, **땡효비**, **뽈라**, **흰둥이**, **빡기**, **현동**, **민당이**... 힘든 공부 속에서 든든한 버팀목이 되어줘서 고맙고, **상만오빠** 나 피자 사줘서 고마워~ 누구보다 우리 **한쓰**... 무슨 말이 필요하겠어~ 이 글을 빌어 정말 마음 깊이 감사하다는 말을 전하고 싶다!

마지막으로 상의 없이 독단적으로 생각하고 결심해서 통보함에도 불구하고 딸을 믿어주시는 **부모님**! 아프지 말고 건강하게만 지내세요! 제가 옆에서 효도할께요! 그리고 누나 말 잘 듣는 우리 잘생긴 **민성**아.. 누나가 너에게 많이 못 해줘서 미안해.. 하지만 누구보다 니가 잘 되길 빈다.. 사랑해♡