



# Epicatechin Prevents Methamphetamine-Induced Neuronal Cell Death via Inhibition of ER Stress

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## Abstract

Methamphetamine (METH) acts strongly on the nervous system and damages neurons and is known to cause neurodegenerative diseases such as Alzheimer's and Parkinson's. Flavonoids, polyphenolic compounds present in green tea, red wine and several fruits exhibit antioxidant properties that protect neurons from oxidative damage and promote neuronal survival. Especially, epicatechin (EC) is a powerful flavonoid with antibacterial, antiviral, antitumor and antimutagenic effects as well as antioxidant effects. We therefore investigated whether EC could prevent METH-induced neurotoxicity using HT22 hippocampal neuronal cells. EC reduced METH-induced cell death of HT22 cells. In addition, we observed that EC abrogated the activation of ERK, p38 and inhibited the expression of CHOP and DR4. EC also reduced METH-induced ROS accumulation and MMP. These results suggest that EC may protect HT22 hippocampal neurons against METH-induced cell death by reducing ER stress and mitochondrial damage.

**Key Words:** Epicatechin, Methamphetamine, Neuroprotection

## INTRODUCTION

Methamphetamine (METH) is an illegal and potent mental stimulant that acts strongly on the central nervous system. METH is abused by more than 35 million people worldwide (Hanson *et al.*, 2004; Tallozy *et al.*, 2008). The serious neuropsychological consequences of METH abuse include memory and cognitive impairment that persist long after withdrawal (Meredith *et al.*, 2005). Repeated METH administration to rats results in long-term damage to sensorimotor cortical neurons, which has been correlated with impaired motor function or cognitive memory (Walsh and Wagner, 1992; Marshall *et al.*, 2007). Since METH has relatively high lipid solubility, the drug permeates through the blood-brain barrier. When METH enters the monoamine reactive end via dopamine or serotonin transporter, it is oxidized instead of vesicular and intracellular dopamine and serotonin, leading to the production of reactive oxygen species and neuronal death (Davidson *et al.*, 2001; Barr *et al.*, 2006).

Recent studies suggest that oxidative stress, mitochondrial apoptosis and excitotoxicity pathways play an important role

in METH-induced neurotoxic damage. Superoxide radicals (Jayanthi *et al.*, 1998) and hydroxyl radicals (Giovanni *et al.*, 1995) were found to mediate the neurotoxicity of METH. Thus, excessive production of free radicals by repeated administration of METH may contribute to oxidative damage (Jayanthi *et al.*, 1998). However, the mechanism associated with METH toxicity has yet to be fully elucidated.

It is well known that METH increases glutamate levels in mammalian brain. High levels of glutamate contribute to neurotoxicity (Eisch *et al.*, 1996; O'Dell and Marshall, 2005). Glutamate induces neuronal apoptosis via interaction between endoplasmic reticulum stress and mitochondrial death pathways upon administration of METH to rodents (Jayanthi *et al.*, 2001, 2004).

Hippocampal neuronal cell death plays an important role in memory disorders associated with various brain diseases (Lee *et al.*, 2012; Debette, 2013). Therefore, preventing hippocampal neuronal cell death represents a new therapeutic strategy to improve memory and cognitive impairment in various neurological disorders. The HT22 hippocampal cell line is functionally deficient in glutamate receptors, and has been

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used to study glutamate-mediated molecular mechanisms (Lee *et al.*, 2012; Kempf *et al.*, 2014). Further, HT22 cells exposed to glutamate exhibit neurotoxicity via oxidative stress such as decreased glutathione, changes in intracellular cysteine homeostasis, inhibition of cysteine uptake, and ultimately activation of ROS resulting in neuronal cell death (Murphy *et al.*, 1989; Stanciu *et al.*, 2000). Oxidative stress and accumulation of ROS eventually lead to hippocampal cell death and affect learning and memory impairment (Yang *et al.*, 2013).

Flavonoids are polyphenolic compounds present in green tea, red wine and several fruits (Cuevas *et al.*, 2009). Flavonoids exhibit antioxidant properties that protect neurons from oxidative damage and promote neuronal survival (Blount *et al.*, 2012). Epicatechin (EC) is a flavonoid with antibacterial (Bhattacharyya *et al.*, 2004), antiviral (Apostolides and Weisburger, 1995), antitumor (Geetha *et al.*, 2004) and antimutagenic effects (Hanasaki *et al.*, 1994) as well as antioxidant effects (Nakagawa and Yokozawa, 2002). It has also been shown to effectively remove nitric oxide and O<sub>2</sub> (Katiyar *et al.*, 1994). In the present study, we investigated the protective effects of EC on METH-induced cytotoxicity using HT22 hippocampal cells. We further investigated whether the cytoprotective effect of EC is involved in oxidative stress regulation.

## MATERIALS AND METHODS

### Materials and reagents

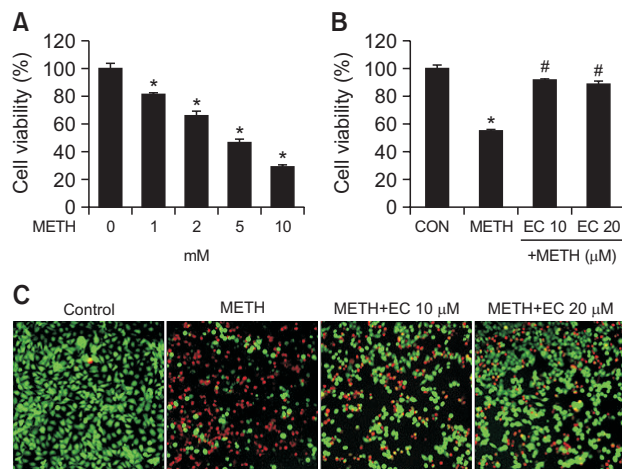
Methamphetamine (METH) was purchased from the Ministry of Food and Drug Safety (Cheongju, Korea). Epicatechin (EC) was purified and received from Dr. Gil-Saeng Jeong, a professor of the College of Pharmacy, Keimyung University (Daegu, Korea). Methamphetamine (METH) was dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) as a 1 M stock solution and stored at 4°C. Further dilution was done in cell culture medium. Antibodies against CHOP, BAX were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, DR4, DR5, cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, cleaved PARP, and  $\beta$ -actin were obtained from Cell Signaling Technology (Beverly, MA, USA).  $\beta$ -actin was used as a loading control.

### Cell line and cell culture

HT22 murine hippocampal neuronal cells (The Salk Institute, La Jolla, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in an atmosphere of 5% CO<sub>2</sub>-95% air. Cells were passaged at 80% confluence in 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid) for 2 min. DMEM, FBS, antibiotic and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA).

### Cell viability assay

HT22 cells were incubated in 24-well plates at a density of  $3 \times 10^5$  cells per well. Media were pretreated with different concentration of Epicatechin (10, 20  $\mu$ M) for 1 h, followed by stimulation with Methamphetamine (1, 2, 5 or 10 mM) for 24 h. After treatment, 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenylterazolium bromide (MTT, 5 mg/ml; Sigma) was added to each well (2.5 mg/mL), following incubation for 4 h at 37°C in a 5% CO<sub>2</sub>. The supernatant was removed, and the formazan pre-



**Fig. 1.** Effects of METH and EC on cell viability in HT22 hippocampal neuronal cells. (A) HT22 cells were treated with various concentrations of METH for 24 h. (B) Cells were pretreated with EC for 1 h, and incubated with 5 mM METH for 24 h. The cell viability was determined by MTT assay and the percent cell viability was plotted as the means  $\pm$  SEM of at least three experiments. (C) Live/Dead viability/cytotoxicity assay depicts the cytotoxic effects of METH and EC in HT22 cells. Fluorescence images of viable cells were stained with calcein-AM (green) and dead cells were stained with ethidium homodimer 1 (red). Percentage of viable cells was calculated under a fluorescence microscope. A total of five random quadrants were selected from each triplicate for quantification. Data are expressed as mean  $\pm$  SEM. \* $p < 0.01$  when compared with untreated control cells. # $p < 0.01$  when compared with METH-treated cells.

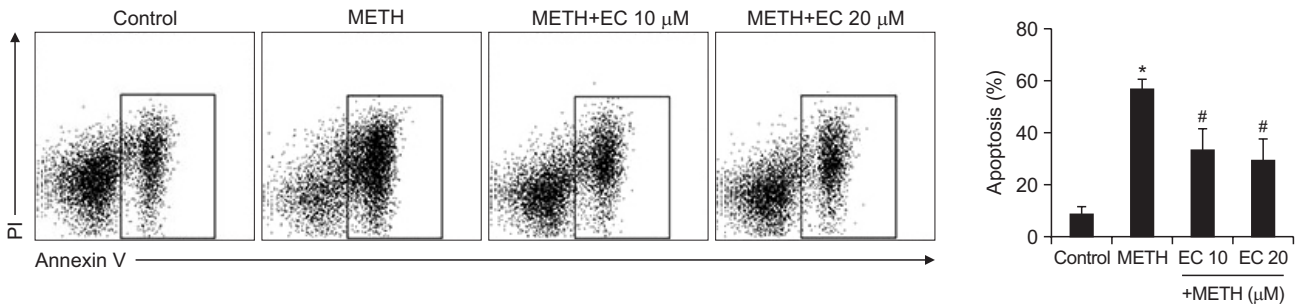
cipitate was dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm using a microplate reader (Tecan Austria GmbH, Salzburg, Austria).

### Live/Dead assay

To measure apoptosis, we used the Live/Dead assay kit (Invitrogen, Gaithersburg, MD, USA), which determines intracellular esterase activity and plasma membrane integrity. Calcein-AM, a non-fluorescent polyanionic dye, is retained by live cells, in which it produces green fluorescence through enzymatic (esterase) conversion. In addition, the ethidium homodimer enters cells with damaged membranes and binds to nucleic acids, thereby producing a red fluorescence in dead cells. Briefly, the cells were seeded in a chamber slide (Nalge Nunc International, Naperville, IL, USA), incubated with 20  $\mu$ M Epicatechin for 1 h, and then treated with 5 mM Methamphetamine for 24 h at 37°C. The cells were stained with the Live/Dead reagent (5  $\mu$ M ethidium homodimer, 5  $\mu$ M calcein-AM) and then incubated at 37°C for 30 min. The cells were analyzed under a Labophot-2 fluorescence microscope (Nikon, Tokyo, Japan).

### Apoptosis analysis

Proportions of apoptotic cells were detected by flow cytometry using the Annexin V-FITC Apoptosis Detection kit I (BD Pharmingen™, BD Biosciences, NJ, USA). The experiment was performed according to the manufacturer's protocol. To investigate the neurotoxic effect of Epicatechin on Methamphetamine, cells were pretreated with 20  $\mu$ M Epicatechin for



**Fig. 2.** Effects of EC on METH-induced apoptosis in HT22 hippocampal neuronal cells. Cells were pretreated with EC for 1 h, and incubated with 5 mM METH for 24 h. Cells were stained with PI and Annexin V, and then evaluated by flow cytometry (left). Percentage of apoptotic cells was detected by flow cytometry (right). Results are shown as mean  $\pm$  SEM from three independent experiments. \* $p$ <0.01 when compared with untreated control cells. # $p$ <0.01 when compared with METH-treated cells.

1 h and then exposed to 5 mM Methamphetamine for 24 h. Floating cells were collected, and the attached cells were trypsinized and washed twice with ice-cold PBS at the indicated time. Then cell suspension ( $1 \times 10^5$  cells/ml) was incubated with Annexin V-FITC for 15 min in the dark. Finally, 400  $\mu$ l of 1X binding buffer was added to each sample. The samples were counted using a BD FACS Verse™ (BD Biosciences) and analyzed by Flowing 2.5 version software.

#### Measurement of intracellular ROS

Intracellular ROS level was measured using the fluorescent dye, DCF-DA. Cells were pretreated with EC (10, 20  $\mu$ M) for 1 h, and treated with 1mM METH for 24 h. Cells were then treated with 5  $\mu$ M of DCF-DA for 15 min at 37°C, and the cells were imaged using fluorescence microscopy.

#### Measurement of MMP

To determine MMP, cells were pretreated with EC for 1 h, followed by treatment with 5 mM METH for 24 h. Cells were washed with warm PBS, resuspended in warm PBS containing 100 nM TMRM, and then incubated at 37°C for 30 min. Cells were washed with warm PBS, resuspended in PBS, and then MMP level was measured by flow cytometry. TMRM fluorescence excited at 488 nm and emitted at 588 nm was measured by FL2 channel using a BD FACS Verse™ (BD Biosciences) and analyzed with Flowjo software. Apoptotic cells showed decreased intensity of TMRM staining. At least ten thousand events were analyzed per sample and the experiment was repeated at least twice.

#### Western blot analysis

The cells were washed with PBS and lysed for 30 min on ice in RIPA lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholate and 5 mM ethylene diamine tetra acetic acid (EDTA)] enriched with a complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), and then centrifuged at 14000 rpm for 10 min at 4°C. Protein concentration was determined by using bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Total protein (30  $\mu$ g) was loaded onto 12% SDS-polyacrylamide gel, separated, and transferred onto polyvinylidene difluoride (PVDF) membrane (Roche, Diagnostics). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 [TBST; 10 mM Tris-HCl (pH 8.0), 150 mM NaCl,

0.05% Tween-20] and incubated with antibody at 4°C. After three washes of 10 min each in TBST, the membranes were incubated with hybridization with horseradish peroxidase conjugated anti-rabbit or anti-mouse secondary antibodies for 2 h and subsequently washed again. The transferred proteins were incubated with super-signal pico-chemiluminescent substrate or dura-luminol substrate (Thermo Scientific, Waltham, MA, USA) for 1 min according to the manufacturer's instructions and visualized with Image Quant™ LAS 4000 (Fujifilm, Tokyo, Japan; Roche Diagnostics).

#### Statistical analysis

Experiments were performed at least three times, with consistent results. The results are given as mean  $\pm$  standard deviation (SD). The  $p$ -value was assessed using ANOVA and Student-Newman-Keul tests. Results were considered statistically significant at  $p$ <0.01.

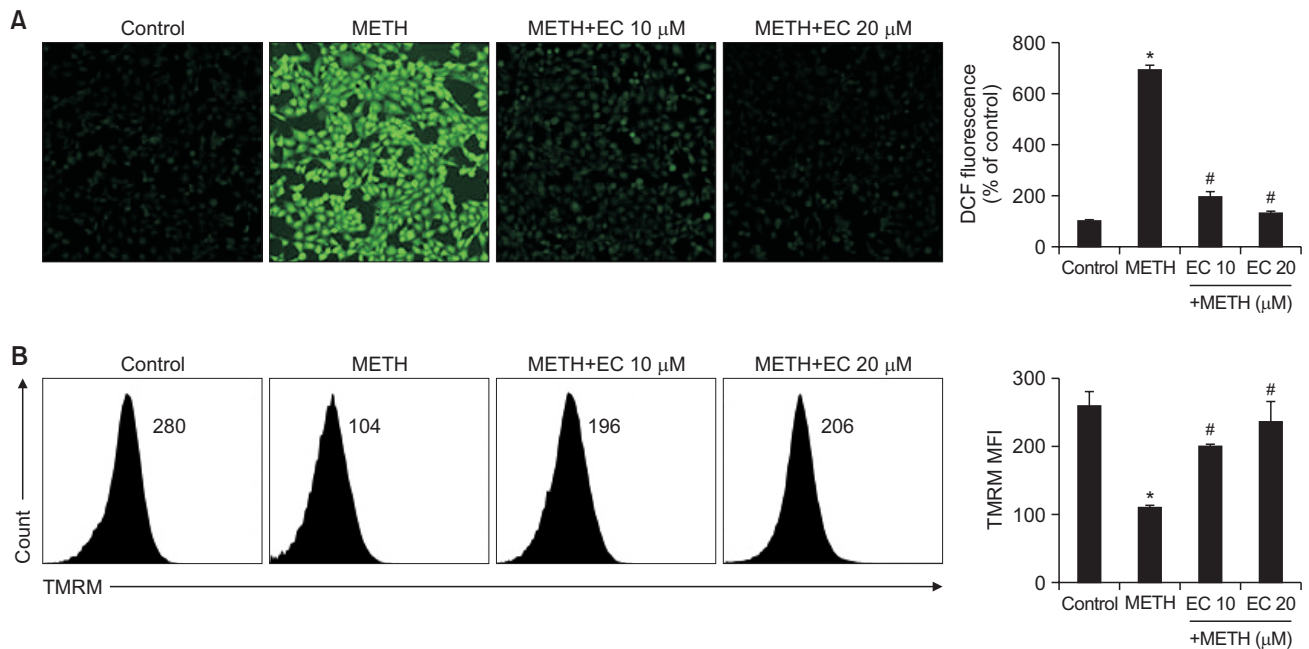
## RESULTS

### EC protects HT22 hippocampal neuronal cells against METH-induced cell death

To determine whether EC was cytoprotective, we measured the cell viability after METH treatment of murine hippocampal HT22 cells. When HT22 cells were treated with various concentrations of METH for 24 h, the cell viability was decreased to 50% at 5 mM (Fig. 1A). To determine the protective effect of EC against METH-induced cytotoxicity, cells were pretreated with 10, 20  $\mu$ M EC for 1 h and incubated with 5 mM METH for 24 h (Fig. 1B). Despite treatment with 5 mM METH, concurrent EC treatment restored the rate of cell survival up to 90%. These results demonstrate that EC protects HT22 hippocampal cells from METH-induced cell death. Further, when we identified live and dead cells using fluorescence dye, the proportion of dead cells was decreased gradually following pretreatment with EC (Fig. 1C).

### EC prevents METH-mediated apoptosis of HT22 cells

Since HT22 cells underwent apoptotic cell death following treatment with METH, we investigated whether EC prevented apoptosis induced by METH. As shown in Fig. 2, analysis of flow cytometry data revealed that METH induced apoptosis of HT22 cells, and co-treatment with EC significantly reduced it. Based on these results, we suggest that EC has a cytoprotective



**Fig. 3.** Effects of EC on METH-induced ROS accumulation and reduction of MMP in HT22 hippocampal neuronal cells. (A) Cells were pretreated with EC for 1 h, and incubated with 1 mM METH for 24 h. Cells were then incubated with 5  $\mu$ M DCF-DA for 15 min. The intracellular ROS level was determined using a fluorescence microscope. (B) Cells were pretreated with EC for 1 h, and treated with 5 mM METH for 24 h. Cells were stained with TMRM and the fluorescence was measured by flow cytometry (left). Percentage of MMP was calculated and plotted in the graph (right). \* $p < 0.01$  when compared with untreated control cells. # $p < 0.01$  when compared with METH-treated cells.

tive effect and prevents neuronal toxicity induced by METH.

### EC reduced ROS accumulation and MMP in HT22 cells

Since oxidative stress including ROS plays an important role in neuronal injury, cell death and neurodegenerative diseases (Simonian and Coyle, 1996), we examined whether ROS mediated METH-induced cytotoxicity. HT22 cells were pretreated with EC for 1 h and exposed to 1 mM METH for 24 h. The ROS levels were analyzed under a microscope via 2',7'-dichlorofluoresce diacetate (DCF-DA) staining. As shown in Fig. 3A, a large amount of ROS accumulation was detected when cells were treated with 1 mM of METH. However, EC significantly inhibited ROS accumulation suggesting that EC has an antioxidative effect.

Next, the mitochondrial membrane potential (MMP) was measured to determine whether the mitochondrial pathway was involved in METH-induced neuronal cell death. As an indicator of mitochondrial damage, we monitored the MMP using tetramethylrhodamine ethyl ester (TMRE). When the cells were incubated with 5 mM METH for 24 h, MMP was significantly reduced; however, pretreatment with EC restored METH-induced MMP reduction (Fig. 3B). Overall, we found that neuronal cytotoxicity by METH was associated with ROS accumulation and MMP collapse, which may be attributed to the interaction between ER stress and mitochondrial death pathway, resulting in increased neuronal death. However, EC inhibited ROS accumulation and protected MMP collapse by METH.

### EC inhibits METH-induced MAPK activation

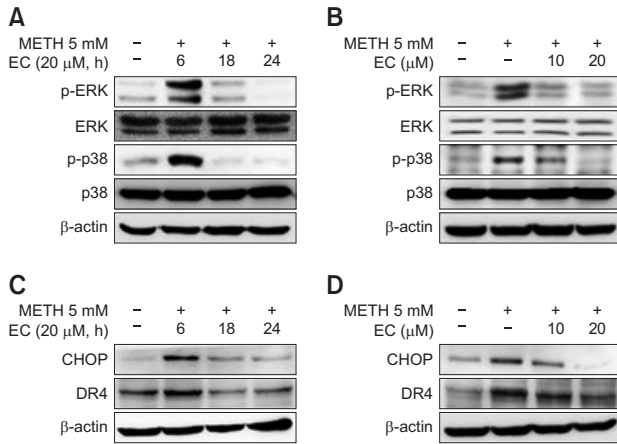
Since MAPKs such as p38, JNK, and ERK mediate cell death by ROS (Ravindran *et al.*, 2011; Son *et al.*, 2013), we

examined whether METH activated MAPKs. The activation of MAPKs was confirmed by Western blot analysis. We further explored whether EC inhibited MAPKs activation mediated by METH. As shown in Fig. 4, EC significantly reduced ERK and p38 activation by METH without affecting JNK (data not shown). The activity of MAPKs was robust until 6 h and eventually declined upon EC exposure (Fig. 4A). The inhibition of MAPKs activity was significantly inhibited at 10  $\mu$ M and 20  $\mu$ M of EC, respectively, and the inhibition was suppressed further at 20  $\mu$ M treatment (Fig. 4B).

Next, we evaluated the expression of CHOP, a marker for ER stress, which is associated with the MAPKs-mediated death pathway. The results showed that METH induced CHOP expression, which was significantly abrogated by EC. Since CHOP is also involved in the expression of death receptor (DR) (Gupta *et al.*, 2013b; Refaat *et al.*, 2014), we examined whether EC affects the expression of death receptor. We found that METH increased the expression of DR4, which was also inhibited by EC treatment. Expression of CHOP and DR4 appears to be elevated until 6 h and eventually declined upon EC exposure (Fig. 4C). The blockade of expression was significantly inhibited further at 20  $\mu$ M treatment (Fig. 4D).

### EC inhibits METH-induced apoptotic pathway

DR4/5 is involved in the extrinsic pathway of apoptosis, whereas the mitochondrial pathway is involved in intrinsic apoptosis (Belt *et al.*, 2014). MMP and DR4 mediate METH-induced cell death (Fig. 4B). Therefore, we investigated whether the cytoprotective effect of EC played a role in both intrinsic and extrinsic apoptotic pathways. As shown in Fig. 5, EC decreased the cleavage of caspase-8 and caspase-3, which are extrinsic apoptotic molecules. EC also reduced the cleavage

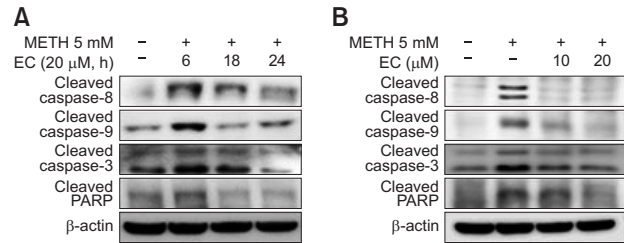


**Fig. 4.** Effects of EC on METH-induced protein expression in HT22 hippocampal neuronal cells. Cells were pretreated with 20  $\mu$ M EC for 1 h, treated with 5 mM METH at various time intervals (A, C), or pretreated with 10  $\mu$ M or 20  $\mu$ M EC for 1 h and then treated with 5 mM METH for 24 h (B, D). Cell lysates were resolved by SDS-PAGE and then analyzed by Western blot using antibodies against p38, p-p38, ERK, p-ERK, CHOP, DR4 and  $\beta$ -actin. The results shown were obtained from at least three independent experiments.

of caspase-9 and PARP, which are signaling molecules in the intrinsic apoptosis. Similar to the results of the previous experiments, METH-induced activation of caspases was decreased 6 h after treatment with EC (Fig. 5A), and the activity was further strongly inhibited at 20  $\mu$ M (Fig. 5B). Collectively, these results suggest that EC protects HT22 cells by preventing METH-induced mitochondrial damage and DR4 pathway.

## DISCUSSION

Since hippocampus plays a key role in drug addiction, several studies analyzed hippocampus in addiction prevention. In particular, hippocampus is one of the brain regions vulnerable to METH (Shimazu *et al.*, 2006; Raudensky and Yamamoto, 2007). METH mediates the interaction between ER stress and mitochondrial death leading to apoptosis of striatal glutamic acid decarboxylase-containing neurons. In addition to neuronal death, astroglial activation may be triggered by METH toxicity (Pubill *et al.*, 2003; Miyatake *et al.*, 2005). Further, METH induces excessive glutamate release, which damages the cortical neurons via an excitotoxic mechanism (Eisch *et al.*, 1996; O'Dell and Marshall, 2005). The toxicity due to oxidative glutamate occurs due to a sharp decline in intracellular glutathione and increased ROS production in HT22 cells, triggering downstream events such as activation of lipoxygenase, increased  $Ca^{2+}$  concentration, and increased nuclear expression of AIF (Pallast *et al.*, 2009). Recent reports suggest that hypoxia-induced excessive ROS and ROS-based ER stress play an important role in the progression of HT22 cell death. Neurons are particularly susceptible to damage by ROS due to their low levels of endogenous antioxidant enzymes and weak antioxidant defense systems (Jia *et al.*, 2013). Thus, oxidative stress induced by ROS contributes to neuronal cell death in the brain. In this study, we investigated the cytoprotective effects of EC on METH-induced oxidative toxicity in



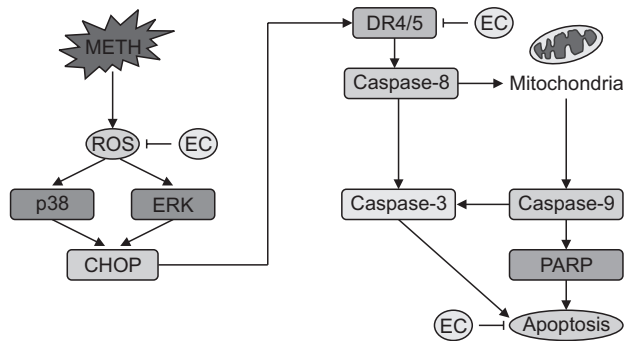
**Fig. 5.** Effects of EC on METH-induced caspases activation in HT22 hippocampal neuronal cells. Cells were pretreated with 20  $\mu$ M EC for 1 h, treated with 5 mM METH at various time intervals (A), or pretreated with 10  $\mu$ M or 20  $\mu$ M EC for 1 h and then treated with 5 mM METH for 24 h (B). Cell lysates were resolved by SDS-PAGE and then analyzed by Western blot using antibodies against cleaved caspase-8, -9, -3, cleaved PARP and  $\beta$ -actin. The results shown were obtained from at least three independent experiments.

hippocampal HT22 cell line.

ER stress induces the release of ROS, which is central to the activation of multiple signaling pathways. METH induces ER stress, and therefore, we determined whether METH exposure generated ROS. The results suggested that METH significantly increased ROS production, which was inhibited by pretreatment with EC (Fig. 3). In addition, we confirmed that EC inhibited the activation of ERK and p38 MAPK and the expression of CHOP and DR4 by METH (Fig. 4). These results indicate that EC inhibits glutamate-induced ER stress by METH. In addition to glutamate, a variety of ER stress inducers, such as tunicamycin, increase CHOP expression and induce cell death by ER stress in HT22 cells (Ono *et al.*, 2012). CHOP is a crucial mediator of ER stress-related apoptosis and is expressed highly in neurodegenerative diseases such as Alzheimer's disease (Ito *et al.*, 2009; Salminen *et al.*, 2009). These studies suggest that the increased expression of CHOP in neurons induces apoptosis associated with ER stress. Therefore, the inhibition of CHOP expression by EC can prevent the effect of ER stress caused by METH (Lauro *et al.*, 2010; Chhunchha *et al.*, 2013). Our study suggests that EC acts as a potent antioxidant against ROS and ER stress-induced apoptosis in HT22 cells.

ER-induced apoptosis increases the expression of DR4 and DR5 via increased expression of CHOP, and ROS is involved in the upregulation of DR4 and DR5. The expression of CHOP and DR4 is upregulated via ERK and p38 MAPK signaling (Park *et al.*, 2010; Sung *et al.*, 2010; Prasad *et al.*, 2011; Gupta *et al.*, 2013a; Yoon *et al.*, 2013). In the present study, we showed that ERK and p38 activities are upregulated by ROS and ER stress induced by METH, resulting in the upregulation of CHOP and DR4 expression. EC significantly abrogated the upregulation.

In general, it is well known that apoptosis occurs via two pathways: the extrinsic pathway mediated via death receptor and the intrinsic pathway via mitochondria. ROS induce apoptosis via both the extrinsic apoptotic receptor and the intrinsic mitochondrial apoptotic pathway. Our study showed that EC inhibited METH-induced activation of caspase-3, -8, -9 and PARP (Fig. 4C). It was also confirmed that EC restored MMP reduced by METH (Fig. 3B). These results suggest that the extrinsic pathway via DR4 and the intrinsic pathway via mitochondria mediate the METH-induced apoptotic pathway, and EC significantly abrogated both apoptotic pathways.



**Fig. 6.** A schematic diagram for cytoprotective effect of EC against METH-induced neuronal cell death.

In this study, we have demonstrated the protective effect of EC against neuronal cell death via oxidative stress and ER stress induced by METH. EC inhibited ROS generation as well as MAPK activity, and CHOP and DR4 expression during METH-mediated apoptosis. In addition, EC inhibits apoptosis via inhibition of caspase activity (Fig. 6). Overall, our results support the hypothesis that EC has a protective effect against METH-induced cytotoxicity in HT22 hippocampal cells. These results provide insight into the etiology of neurodegenerative diseases associated with METH abuse.

## ACKNOWLEDGMENTS

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