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Chloroacetic acid induced neuronal cells death through oxidative stress-mediated p38-MAPK activation pathway regulated mitochondria-dependent apoptotic signals

Chun-Hung Chen[a,b,1], Sz-Jie Chen[a,1], Chin-Chuan Su[c,1], Cheng-Chieh Yen[d,e,1], To-Jung Tseng[f,1], Tzyy-Rong Jinn[g,1], Feng-Cheng Tang[h], Kuo-Liang Chen[i], Yi-Chang Su[g], kuan-I Lee[j], Dong-Zong Hung[k,**], Chun-Fa Huang[g,*]

[a] School of Pharmacy, College of Pharmacy, China Medical University, Taichung 404, Taiwan
[b] Department of Emergency, China Medical University Hospital, Taichung 404, Taiwan
[c] Department of Otorhinolaryngology, Head and Neck Surgery, Changhua Christian Hospital, Changhua City 500, Taiwan
[d] Department of Occupational Safety and Health, College of Health Care and Management, Chung Shan Medical University, Taichung 402, Taiwan
[e] Department of Occupational Medicine, Chung Shan Medical University Hospital, Taichung 402, Taiwan
[f] Department of Anatomy, College of Medicine, China Medical University, Taichung 404, Taiwan
[g] School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 404, Taiwan
[h] Department of Occupational Medicine, Changhua Christian Hospital, Changhua City 500, Taiwan
[i] Department of Urology, China Medical University Hospital, and School of Medicine, China Medical University, Taichung 404, Taiwan
[j] Department of Emergency, Buddhist Tzu Chi General Hospital, Taichung Branch, Taichung 427, Taiwan
[k] Division of Toxicology, Trauma & Emergency Center, China Medical University Hospital, No. 2 Yude Rd., Taichung 404, Taiwan

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ABSTRACT

Chloroacetic acid (CA), a toxic chlorinated analog of acetic acid, is widely used in chemical industries as an herbicide, detergent, and disinfectant, and chemical intermediates that are formed during the synthesis of various products. In addition, CA has been found as a by-product of chlorination disinfection of drinking water. However, there is little known about neurotoxic injuries of CA on the mammalian, the toxic effects and molecular mechanisms of CA-induced neuronal cell injury are mostly unknown. In this study, we examined the cytotoxicity of CA on cultured Neuro-2a cells and investigated the possible mechanisms of CA-induced neurotoxicity. Treatment of Neuro-2a cells with CA significantly reduced the number of viable cells (in a dose-dependent manner with a range from 0.1 to 3 mM), increased the generation of ROS, and reduced the intracellular levels of glutathione depletion. CA also increased the number of sub-G1 hypodiploid cells; increased mitochondrial dysfunction (loss of MMP, cytochrome *c* release, and accompanied by *Bcl-2* and *Mcl-1* down-regulation and *Bax* up-regulation), and activated the caspase cascades activations, which displayed features of mitochondria-dependent apoptosis pathway. These CA-induced apoptosis-related signals were markedly prevented by the antioxidant *N*-acetylcysteine (NAC). Moreover, CA activated the JNK and p38-MAPK pathways, but did not that ERK1/2 pathway, in treated Neuro-2a cells. Pretreatment with NAC and specific p38-MAPK inhibitor (SB203580), but not JNK inhibitor (SP600125) effectively abrogated the phosphorylation of p38-MAPK and attenuated the apoptotic signals (including: decrease in cytotoxicity, caspase-3/-7 activation, the cytosolic cytochrome *c* release, and the reversed alteration of *Bcl-2* and *Bax* mRNA) in CA-treated Neuro-2a cells. Taken together, these data suggest that oxidative stress-induced p38-MAPK activated pathway-regulated mitochondria-dependent apoptosis plays an important role in CA-caused neuronal cell death.

Abbreviations: CA, chloroacetic acid; ROS, reactive oxygen species; GSH, glutathione; MMP, mitochondrial membrane potential; NAC, *N*-acetylcysteine; MAPK, mitogne-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-related kinase.

* Corresponding author. Tel.: +886 4 22053366x3323; fax: +886 4 22333641.
** Corresponding author. Tel.: +886 4 22052121x7612; fax: +886 4 22333076.
E-mail addresses: dzhung@mail.cmu.edu.tw (D.-Z. Hung), cfhuang@mail.cmu.edu.tw (C.-F. Huang).
[1] These authors contributed equally to this work.

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1. Introduction

Chemicals of natural or manufacturing origin by human activities exist in the mammalian environment. Humans are exposed to these chemicals through occupational exposure during produced processes, environmental exposure to toxic waste, and exposure to contaminated food/products (Pruss-Ustun et al., 2011). The growing number of studies have been reported that chemicals or environmental risk factors can trigger the multitude of deleterious reactions, which lead to cell death and/or organ injuries that are closely related to development of diseases, including cancer and neurodegenerative disorders (Dosunmu et al., 2007; Mates et al., 2010).

Chloroacetic acid (CA), a toxic chlorinated analog of acetic acid and 25–40 times more toxic than acetic, dichloroacetic, or trichloroacetic acids, is an important chemical used industrially as an herbicide, detergent, and disinfectant, and as the chemical intermediates during the synthesis of various products such as vitamin A, caffeine, and dyes (Budavari, 1989; U.S. Environmental Protection Agency, 1988). Thousands of industrial and agricultural workers are occupationally exposed to CA due to its widespread usage. In the United States and other countries, CA is one of the most commonly by-products formed during the chlorination processes for disinfection of drinking water and also produced in the environment as the metabolites of other widely used chemicals such as 1,2-dichloroethane, vinyl chloride, and vinylidene chloride, which lead to human chronically expose (Hathway, 1977; Krasner et al., 1989; USEPA, 2002; Yllner, 1971). Several studies in the clinical cases and animal experiments have indicated that CA not only highly corrosive to contact tissues (liver, kidney, lung, and skin), but also can induce histopathological changes and organic dysfunction, and even cause systemic death by accidental poison and various routes exposure (Bhat et al., 1991; Daniel et al., 1991; Dote et al., 2003; Kusch et al., 1990; Rogers, 1995). Quick et al. (1983) has reported that the neurotoxic properties of CA at higher acute doses, and Berardi et al. (1987) also have found serious impairment of the blood-brain barrier (BBB) function in mice treated with CA. However, the mechanism underlying the toxicity of CA mostly remains to be clarified, specifically in neurotoxicity.

Apoptosis, known as programmed cell death, is a form of cell death that the pathologically changed into phenomenon to eliminate damaged cells in various situations and to maintain the balance between cell death and cell proliferation (Desjardins and Ledoux, 1998; Kerr, 2002). A series of distinctive and characteristic changes, including cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation are observed during apoptosis. Many toxic insults induce cell death in mammalian through apoptosis (Chen et al., 2010; Tang et al., 2009). Apoptosis generally occurs via two basic pathways: intrinsic and extrinsic pathway. The intrinsic pathway is triggered by proapoptotic signals that result in disruption of mitochondrial functions leading to cell death, which indicate that mitochondria play an important role for execution of apoptosis (Kroemer et al., 1997). The extrinsic pathway involves the initiation of apoptosis through ligation of cell surface death receptors (such as tumor necrosis factor receptor (TNFR) members) located on the plasma membrane; therefore, this pathway is also known as death receptor pathway (Nagata, 1997). On the other hand, many studies have established a relation between a chemical or toxic reagents-induced apoptosis and oxidative stress damage. Because a large number of evidences indicate that reactive oxygen species (ROS) formed in the exposure to chemical or toxic reagents could be responsible for those toxic effects in mammalian cells and tissues (Kitamura and Hiramatsu, 2010; Lu et al., 2011a,b). Oxidative stress is the state as a disturbance in the pro-oxidant-antioxidant balance that causes production of free radical leading to cell damage. Oxidative stress has been implicated in the several pathophysiological processes such as carcinogenesis, inflammation, aging, and the development of diseases, like chronic obstructive pulmonary disease, neurodegenerative disease (Emerit et al., 2004; Spector, 2000). Further, apoptosis has been closely linked to these diseases, which suggests that both oxidative stress and apoptosis may be involved in the pathophysiology of these diseases (Kannan and Jain, 2000). Recently, growing literatures have indicated that oxidative stress-mediated DNA damage leading to apoptosis seems to be the predominant pathway of neuronal cells death in the brain of Alzheimer's disease (AD) (Loh et al., 2006; Perry et al., 2000). Although some studies have been proposed for ROS-induced neuronal cell apoptosis, integrated and detailed mechanisms are yet to be clarified.

Mitogne-activated protein kinase (MAPK) signaling transduction pathway, consists of three subfamilies: c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK) 1/2, and p38 MAPK, is known to be activated by oxidative stress (Son et al., 2011). MAPKs, a family of serine-threonine protein kinases, play a critical role in a variety of cellular activities such as proliferation, differentiation, transformation, or response to environmental stimuli (survival and death) (Chang and Karin, 2001; Cargnello and Roux, 2011). Deviation from the strict regulation of MAPK signaling pathways by oxidative stress causes to development of human diseases, including various types of cancer, diabetes mellitus, and neurodegenerative diseases (Kim and Choi, 2010; Loh et al., 2006; Lopes et al., 2008). Recently, the growing studies have also indicated that toxic insults activate MAPK signaling pathways via oxidative stress, which implicate the involvement of toxic insults in neuronal cell injuries or apoptosis (An et al., 2011; Chen et al., 2009; Yen et al., 2011). Although, more and more investigation attempt to explore the cellular pathway of oxidative stress, which leads to apoptosis in neuronal cells, the molecular mechanisms and an important role underlying the effect of CA-induced oxidative stress-mediated activation of the MAPKs pathway in neuronal cell apoptosis is not yet clarified.

In this study, we tried to investigate the role of CA-induced oxidative stress-mediated activation of the MAPKs pathway leading to apoptosis in neuronal cells. To this issue, we sought to examine the cytotoxic effects and mechanisms by which CA induced the generation of ROS and the depletion of glutathione (GSH), the disruption of mitochondrial function, the activations of caspase cascades, and the phosphorylation of JNK/ERK1/2/p38-MAPK in neuro-2a cells. Furthermore, the potential protective effects of antioxidant NAC, SP600125 (specific JNK inhibitor), and SB203580 (specific p38-MAPK inhibitor), which used at different stages to confirm the involvement of major signal pathways on CA-induced Neuro-2a cell death, were also investigated.

2. Materials and methods

2.1. Materials

Unless specified, otherwise, all chemicals and laboratory plastic wares were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Falcon Labware (Bectone-Diskinson, Franlin Lakes, NJ, USA), respectively. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (Pen-Strep®) were obtained from Gibco (Gibco BRL, Life Technologies, USA). Commercial LPO assay kit was purchased from Calbiochem (San Diego, USA). 2′, 7′-dichlorofluorescin diacetate (DCFH-DA), and 3,3′-dihexyloxacarbocyanine iodide (DiOC6) were purchased from Molecular Probes (Eugene, OR, USA). Bicinchoninic acid protein assay kit was purchased from Pierce (Pierce, Rockford, IL, USA). Mouse- or rabbit-monoclonal antibodies specific for caspase-3, -7, -9, and poly (ADP-ribose) polymerase (PARP), cytochrome *c*, phospho-JNK, phospho-p38 and phospho-ERK1/2, anti-JNK-1, p38, ERK1/2, and α-tubulin were purchased from Cell Signaling Cell Signaling Technology, Inc., USA).

2.2. Cell culture

Murine neuroblastoma Neuro-2a cells were purchased from American Type Culture Collection (CCL-131, American Type Culture Collection (ATCC), Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cell cultures were maintained in plastic tissue culture dish in a humidified chamber with a 5% CO_2–95% air mixture at 37 °C. Cells were seeded to 6-, 12-, or 24-well culture plates for each experiment and allowed to grow for 12–18 h prior to treatment with CA (final pH. state about 7.4–7.6) for different time intervals.

2.3. Determination of cell viability

Cells were washed with fresh media and cultured in 96-well plates (2×10^4 cells/well) and then treated with CA in the absence or present (1 h pre-treatment) of NAC (2.5 mM), SP600125 (specific JNK inhibitor), and SB203580 (specific p38-MAPK inhibitor) for 24 h. At the end of treatment, the medium was aspirated and cells were incubated with fresh medium containing 0.2 mg/mL 3-(4,5-dimethyl thiazol-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT) was added. After 4 h, the medium was removed and the blue formazan crystals were dissolved in 100 μL dimethyl sulfoxide (DMSO). Following mixing, absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Bio-tek μQuant Monochromatic Microplate Spectrophometer, MTX Lab Systems, Inc.).

2.4. ROS production

ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe: DCFH-DA, as described Chen et al. (2010). In brief, cells were seeded at 2×10^5 cells/well in a 24-well plate and incubated with CA. At the end of various time course treatments, cells were incubated with medium containing with 20 μM DCFH-DA for 15 min at 37 °C. DCFH-DA was converted by intracellular esterases to 2′,7′-dichlorofluorescin (DCFH). In the presence of H_2O_2 or other proper oxidant, DCFH was oxidized into the highly fluorescent 2′,7′-dichlorofluorescein (DCF). After incubation with the dye, cells were harvested and washed twice with PBS, and then re-suspended in ice-cold phosphate buffered saline (PBS) in a dark environment. The intracellular peroxide levels were measured by flow cytometer (FACScalibur, Becton Dickinson, Sunnyvale, CA), that emitted a fluorescent signal at 525 nm.

2.5. Analysis of intracellular glutathione contents

Neuro-2a cells were seeded at 2×0^5 cells/well in a 24-well plate and incubated with CA in the absence or present (1 h pre-treatment) of NAC (2.5 mM). At the end of treatment (for 8 h), cells were incubated medium containing with 60 μM monochlorobimane (mBCL, a fluorescent probe for determining the intracellular GSH levels) for further 30 min at 37 °C. After loading the cells with mBCL, the supernantants were discarded, and cells were washed twice with PBS and then lyzed in lysing buffer (10 mM Tris, 0.25 M sucrose in 0.05% Triton X-100, pH 7.5). The mBCL-GSH related fluorescent intensity of the intracellular fraction was monitored by a fluorescence spectrometer (Gemini XPS Microplate Reader, Molecular Devices, USA) with excitation and emission wavelengths of 385 and 485 nm (Huang et al., 2012).

2.6. Flow cytometry analysis of apoptotic cells

2.6.1. Detection of sub-G1 DNA content

Neuro-2a cells were seeded and incubated with CA in the same manner as for Section 2.4 analysis for 24 h at 37 °C. At the end of treatment, cells were harvested and washed with PBS, then re-suspended in 1 mL of cold 70% (v/v) ethanol, and stored at 4 °C for 24 h. Then, the fixed cells were washed twice with PBS and incubated at 37 °C for 30 min with 10 μg/mL ribonuclear (Rnase) dissolved in 0.5 mL of 0.2% Triton X-100/PBS solution. Following the incubation, cells were washed with PBS and stained with 50 μg/mL propidium iodide (PI) at 4 °C for 30 min in dark condition. The Stained cells were subjected to flow cytometry analysis of DNA content (FACScalibur, Becton Dickinson). Nuclei displaying hypodiploid, sub-G1 DNA contents were identified apoptosis.

2.6.2. Determination of phosphatidyl serine externalization: annexin-V fluorescein Isothiocyanate (FITC) binding assay

The externalization of phosphatidylserine is an early event in apoptosis. Flow cytometry analysis was performed to determine this event using the annexin-V-FITC binding assay kit (BioVision). Cells were seeded and incubated with CA in the same manner as for Section 2.4 analysis for 24 h at 37 °C. Then, the cells were washed twice with PBS and stained with annexin-V-FITC for 20 min at room temperature. Following the incubation, cells were washed twice PBS, and the apoptosis level was determined by measuring the fluorescence of the cells by flow cytometry analysis (FACScalibur, Becton Dickinson).

2.6.3. Detection of caspase 3/7 activity

Caspase 3/7 is the widely accepted as a reliable indicator for cell apoptosis since the activation of caspase 3/7 (Kasof and Gomes, 2001). The FLICA DEVD-FMK caspase 3/7 assay kit (Immunohistochemistry Technologies, LCC.) was used to determine apoptosis by flow cytometry analysis. In brief, cells were seeded and incubated with CA in the same manner as for Section 2.4 analysis for 24 h at 37 °C. At the end of treatment, cells were harvested to 1.5 mL eppendrof and washed with PBS, and stained with fluorescence probe for 10 min in dark condition at room temperature. After incubation with the dye, the fluorescent intensity of cells was analyzed by a flow cytometer (excitation at 488 nm and emission at 515 ± 20 nm; FACScalibur, Becton Dickinson).

2.7. Analysis of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was indicated by retention of the dye: $DiOC_6$, which localized to mitochondria, and the reduced accumulation of $DiOC_6$ reflected the loss of the mitochondrial permeability transition. Briefly, cells were seeded and incubated with CA in the same manner as for Section 2.5 for 6 and 24 h at 37 °C. At the end of treatments, cells were incubated with medium with 100 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) for 30 min at 37 °C. After incubation with the dye, cells were harvested and washed twice with PBS, and then re-suspended in ice-cold PBS. MMP was analyzed by FACScan flow cytometer (Becton Dickinson) (Huang et al., 2012).

2.8. Western blot analysis

Neuro-2a cells were seeded at 1×10^6 cells/well in a 6-well plate and incubated with CA in the same manner as for Section 2.3 analysis at 37 °C. At the end of various time course treatments, the expressions of protein activation were analyzed by the protocol performance of Western blot (as described previously by Chen et al., 2010; Lu et al., 2011b). In brief, equal amounts of proteins (50 μg per lane) was subjected to electrophoresis on 10% (W/V) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (**PVDF**) membranes. The membranes were blocked for 1 h in PBST (PBS, 0.05% Tween-20) containing 5% nonfat dry milk. After blocking, the membranes were incubated with specific antibodies against caspase-3, -7, -9, and poly(ADP-ribose) polymerase (PARP), phospho-JNK, phospho-p38, phospho-ERK1/2, JNK-1, p38, ERK1/2, and α-tubulin in 0.1% PBST (1:1000) for 1 h. After they were washed in 0.1% PBST followed by two washes (15 min each), the respective secondary antibodies conjugated to horseradish peroxidase were applied for 1 h. The antibody-reactive bands were revealed by enhanced chemiluminescence reagents (Perkin-Elmer™, Life Sciences) and were exposed on the Fuji radiographic film. When the detection of cytosol cytochrome *c* expression was performed as previously described in Chen et al. (2010). In brief, at the end of treatments, cells were detached, washed twice with PBS, and then homogenized with a pestle and mortar in the extract buffer (0.4 M mannitol, 25 mM MOPS (pH 7.8), 1 mM EGTA, 8 mM cysteine, and 0.1% (w/v) bovine serum albumin). The cell debris was removed via centrifugation at 6000 × *g* for 2 min. The supernatant was recentrifuged at 12,000 × *g* for 15 min to pellet the mitochondria. The supernatant (cytosolic fraction) was detected cytochrome *c* expression by Western blotting analysis.

2.9. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), and complementary DNA (cDNA) was synthesized from the messenger RNA (mRNA) using the AMV RTase (reverse transcriptase enzyme, Promega Corporation, Pty. Ltd.). Quantitative real-time PCR (qPCR) analysis was performed using Taqman® one-step PCR Master Mix (Applied Biosystems, Foster City CA). Briefly, total cDNA (100 ng) was added per 25 μL reaction buffer with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (β-actin, the housekeeping gene, was used as an internal control). Quantitative RT-PCR assays were carried out in triplicate on StepOnePlus sequence detection system. Cycling conditions were 10 min of polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as CT). Prior to conducting statistical analyses, the fold change from the mean of control group was calculated for each individual sample.

2.10. Statistical analysis

Data are presented as means ± standard deviation (S.D.). The significance of difference was evaluated by the Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA) was used for analysis, and the Duncans's post hoc test was applied to identify group differences. The *p* value less than 0.05 was considered to be significant.

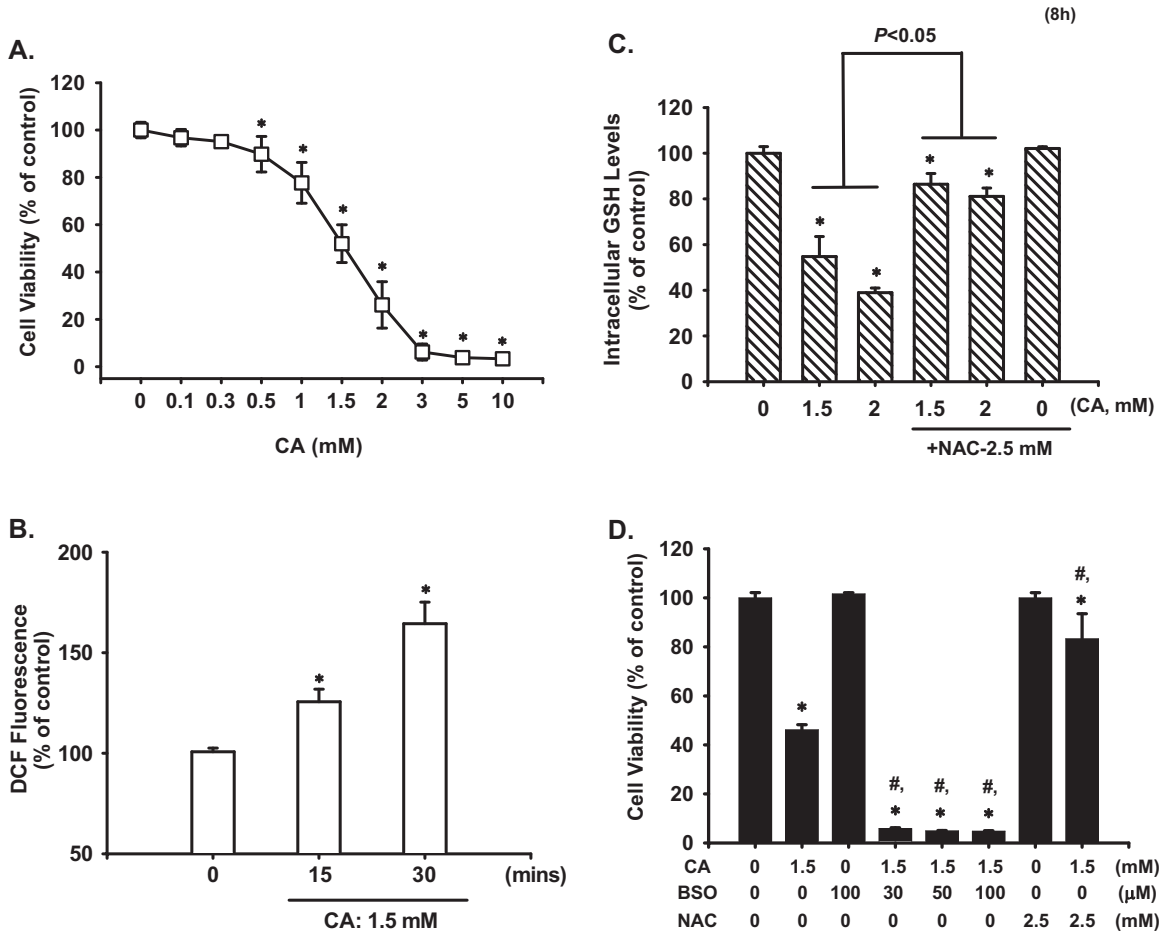

Fig. 1. Effects of chloroacetic acid (CA) on cell viability and oxidative stress damage generation in Neuro-2a cells. (A) Cells were treated with CA (0.1–10 mM) for 24 h, and cell viability was determined by MTT assay. (B) Neuro-2a cells were treated with CA (1.5 mM) for 15 and 30 min, and the levels of ROS were determined by flow cytometry. (C) Cells were treated with CA (1.5 and 2 mM) in the absence or presence of the anti-oxidant NAC (2.5 mM), and the intracellular glutathione (GSH) levels (for 8 h) were detected using a sensitive fluorescent probe mBCL. (D) Neuro-2a cells were treated with CA (1.5 mM) for 24 h in the absence or presence of buthionine sulfoximine (BSO; 30, 50, and 100 μM, pretreatment for 1 h), and cell viability was determined by MTT assay. Data are presented as mean ± S.D. for four independent experiments with triplicate determination. $^{*}p < 0.05$ as compared with vehicle control. $^{\#}p < 0.05$ as compared with CA alone.

3. Results

3.1. Effects of chloroacetic acid on cell viability, ROS production and intracellular GSH depletion in Neuro-2a cells

To investigate the cytotoxicity of chloroacetic acid-induced in neuron cells, we performed the MTT assay to determine the viability of Neuro-2a cells. The viability of Neuro-2a cells was significantly reduced after treatment with CA for 24 h in a dose-dependent manner (a range from 0.1 to 10 mM), and the median lethal dose (LD_{50}) was approximately 1.5 mM (Fig. 1A).

Next, to examine the effect of CA on oxidative stress damage, we treated the Neuro-2a cells with CA and measured the levels of ROS and intracellular GSH. As shown in Fig. 1B, exposure of cells to CA (1.5 mM) for 15 and 30 min significantly increased the intracellular ROS levels, which were determined by flow cytometry using DCF fluorescence probe as an indicator of ROS formation. Incubation of Neuro-2a cells to CA (1.5 and 2 mM) for 8 h significantly reduced the intracellular GSH levels by using an mBCl fluorescent probe, which could be reversed by 2.5 mM NAC (1.5 mM CA, 54.72% ± 8.78%, with NAC, 86.41% ± 4.72% of control; 2.0 mM CA, 38.96% ± 3.49%, with NAC, 81.12% ± 3.64% of control, $n = 8$, $p < 0.05$ as compared with CA alone) (Fig. 1C). Furthermore, pretreatment with buthionine sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis, for 1 h followed by exposure to 1.5 mM CA for 24 h induced severe cytotoxicity as observed by a marked reduction in the number of viable cells (Fig. 1D). In addition, CA-induced reduction in the viability of Neuro-2a cells could be reversed by treatment with NAC (1.5 mM CA, 51.99% ± 7.98%, with NAC, 77.64% ± 6.04% of control; 2 mM CA, 26.11% ± 9.80%, with NAC, 64.64% ± 8.34% of control, $n = 8$; $p < 0.05$ as compared with CA alone).

3.2. CA-induced apoptosis, mitochondrial dysfunction, and caspase cascades activation in Neuro-2a cells

In order to ascertain whether CA-induced cytotoxicity through an apoptotic mechanism, we analyzed the population of sub-G1 hypodiploid cells (using a fluorescent probe: PI) and the membrane externalization of phosphatidylserine (PS) (using an annexin V-FITC binding assay) by flow cytometry. As shown in Fig. 2, Neuro-2a cells that were treated with CA for 24 h triggered the increase in the sub-G1 hypodiploid cell population in a dose-dependent manner (a range from 0.5 to 2 mM) (Fig. 2A) and also remarkably induced the annexin V-FITC binding fluorescent intensity after treatment of cells with CA (Fig. 2B), which could be reversed by NAC (2.5 mM).

To further investigated whether CA-induced apoptosis is mediated through mitochondrial dysfunction, the loss of mitochondrial membrane potential was determined by flow cytometry using a

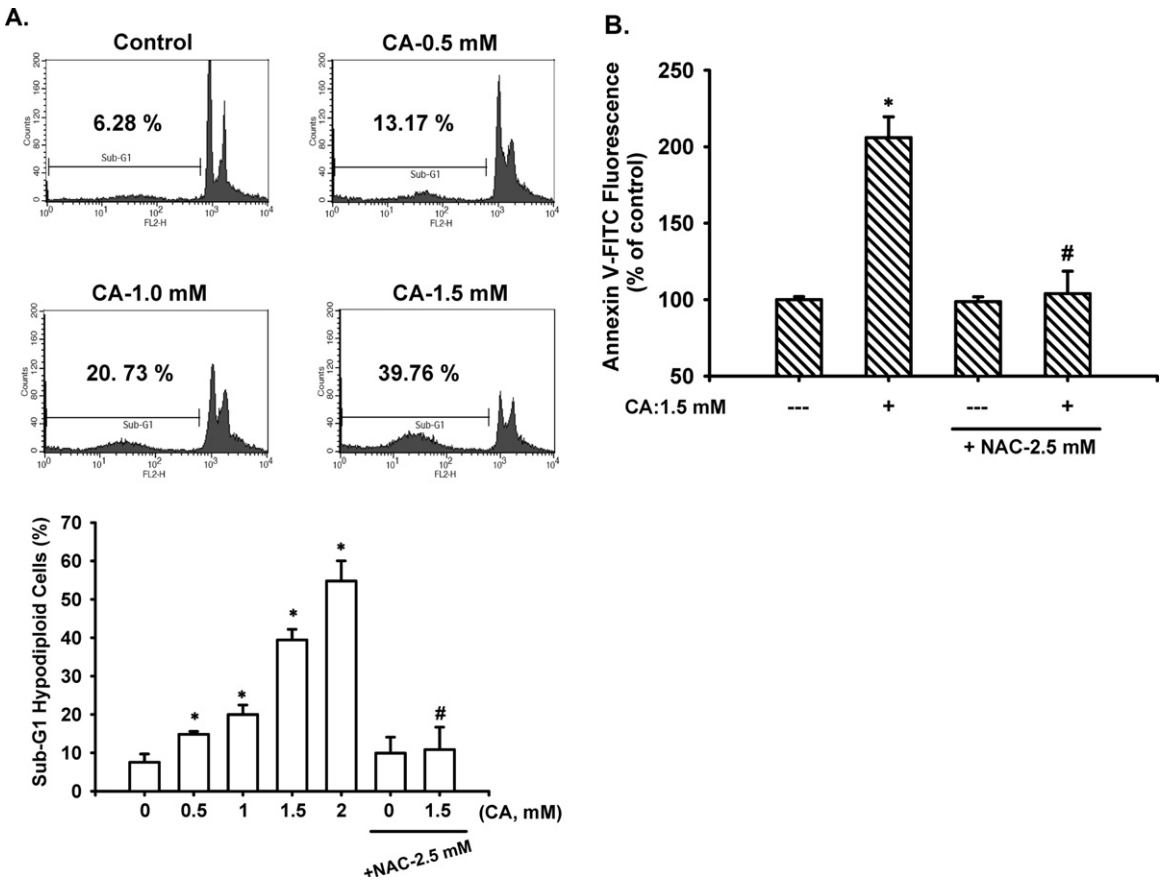

Fig. 2. Chloroacetic acid (CA) induced apoptosis in Neuro-2a cells. Cells were treated with CA (0.5-2 mM) for 24 h in the absence or presence of NAC (2.5 mM), and (A) sub-G1 hypodiploid population (genomic DNA fragmentation) and (B) phosphatidylserine exposure on the outer cellular membrane leaflets by staining with annexin V-FITC fluorescent probe were measured using flow cytometry analysis. Data are presented as mean ± S.D. for four independent experiments with triplicate determination. $^{*}p < 0.05$ as compared with vehicle control. $^{\#}p < 0.05$ as compared with CA alone.

mitochondrial cationic dye, $DiOC_6$. As shown in Fig. 3A, exposure of Neuro-2a cells to CA (1.5 mM) for 6 h revealed a light, but statistically significant depolarization of the MMP and induced a greater loss of MMP after 24 h treatment. Next, we examined the release of cytochrome c from the mitochondria into the cytosol in CA-treated Neuro-2a cells. Exposure of cells to CA (1.5 mM) for 6 h effectively increased cytochrome c release in the cytosolic fraction, and this effect was more significantly after 24 h CA exposure (Fig. 3B). Moreover, we explored the change in the expression of genes belonging to the Bcl-2 family. Treatment of Neuro-2a cells with CA (1.5 mM) significantly induced the mRNA expression of pro-apoptotic *Bax* (1.85 ± 0.25 fold of control; $p < 0.05$), and this change was accompanied with dramatic decreases in the mRNA expressions of anti-apoptotic *Bcl-2* and *Mcl-1* (0.45 ± 0.07 and 0.78 ± 0.06 fold of control; $p < 0.05$) (Fig. 3C). Thus, CA induced a significant shift in the pro-apoptotic/ant-apoptotic Bcl-2 ratio toward a state associated with apoptosis.

Furthermore, one of the characteristics of the apoptotic process is the activation of cysteine proteases, which play the essential role in both initiators and executors of apoptosis (Yuan and Yankner, 2000). As shown in Fig. 4, exposure of Neuro-2a cells to CA (1.5 mM) for different time intervals (4–24 h) induced the significant expressions of cleaved caspase-3 (17/19-kDa), caspase-7 (20-kDa), and caspase-3/-7 activity. On the other hand, CA also markedly increased the protein expression of 20-kDa cleaved caspase-7 and 89-kDa cleaved product (active form) of PARP. Upstream caspase-9 activation increased significantly, as shown that treatment with CA caused the degradation of 51-kDa procapase-9 and increase of 37/39-kDa cleaved caspase-9 levels (Fig. 4A). These CA-induced responses could be reversed by pretreatment with 2.5 mM NAC (Figs. 3 and 4). Therefore, these results implicate that the mitochondria-dependent apoptotic pathway involves in CA-induced ROS production causing Neuro-2a cell death.

3.3. *Effects of CA on activation of MAPK signaling pathway in Neuro-2a cells*

MAPK plays a crucial role in a variety of toxic insults-induced apoptotic signaling pathway (Cowan and Storey, 2003). Therefore, an important role of MAPKs in CA-induced neuron cell apoptosis was needed to investigate. As shown in Fig. 5A, treatment of Neuro-2a cells with 2.5 mM CA for 15–120 min significantly increased the phosphorylation levels of JNK and p38 MAPK, but not that ERK1/2. The effects of CA on the activation of JNK and p38-MAPK could be abrogated by NAC (for 1 h pretreatment), which indicated that ROS play a key role in CA induced JNK and p38 MAPK activation. To further elucidate the relationship between CA induced Neuro-2a cells apoptosis and JNK and p38 MAPK activation, the cells were pretreated with the specific JNK inhibitor (SP600125, 20 μM) and p38-MAPK inhibitor (SB203580, 20 μM) for 1 h and then incubated with CA (1.5 mM). It was revealed that CA induced the increase in the phosphorylation levels of JNK and p38 MAPK proteins was remarkably prevented by JNK and p38-MAPK inhibitor (Fig. 5B, *a* and *b*). However, only p38-MAPK inhibitor, but not that JNK inhibitor, could effectively attenuated CA induced neuron cell cytotoxicity (Fig. 6A) and the increase in caspase-3/-7 activity (Fig. 6B), the sub-G1 hypodiploid cell population (Fig. 6C), and apoptotic cells

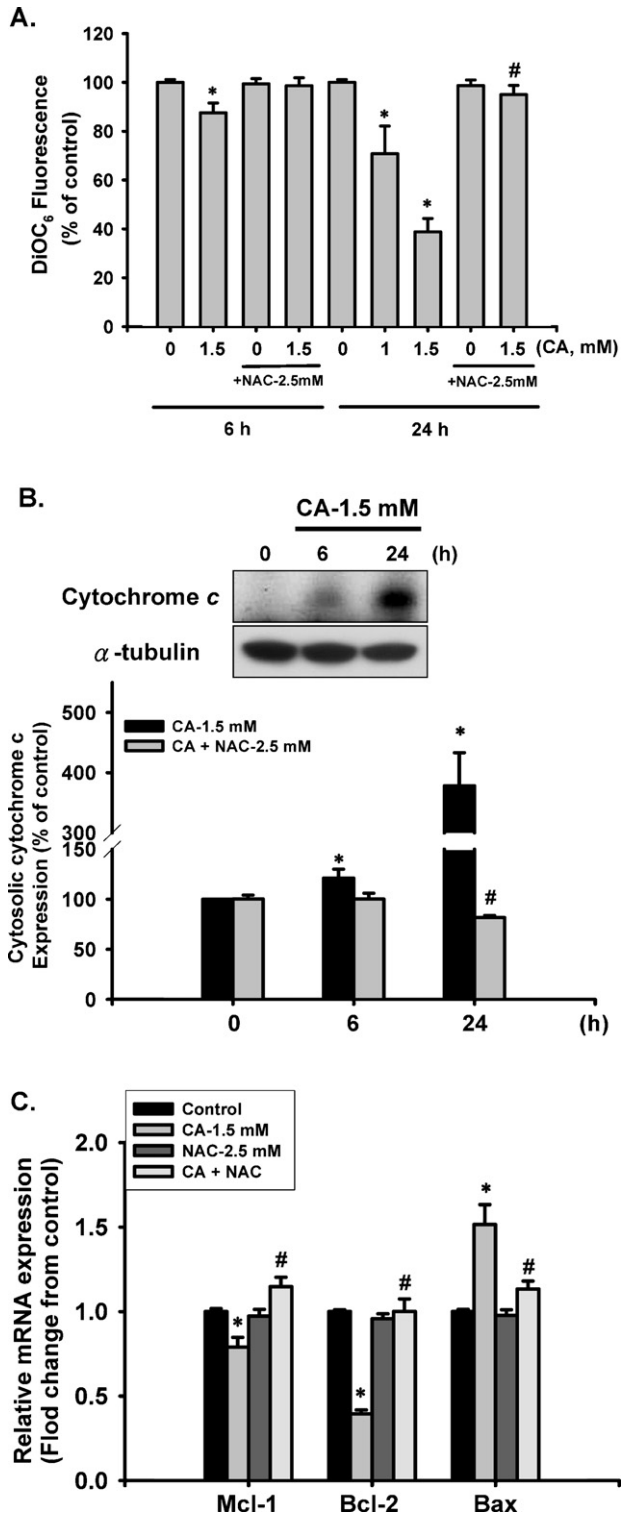

Fig. 3. Chloroacetic acid (CA) induced mitochondrial dysfunction in Neuro-2a cells. Cells were treated with CA (1 or 1.5 mM) for 8 and 24 h in the absence or presence of NAC (2.5 mM). (A) Mitochondrial membrane potential (MMP) depolarization was determined by flow cytometry, (B) cytosolic cytochrome c level was examined by Western blot analysis, and (C) the expression of anti-apoptotic (*Mcl-1* and *Bcl-2*) and pro-apoptotic (*Bax*) genes was analyzed by real-time quantitative RT-PCR. Data in A and C are presented as mean ± S.D. for four independent experiments with triplicate determination. Result shown in B, α-tubulin is used as an internal control, and expression of cytosolic cytochrome c protein is quantified by densitometric analysis at least three independent experiments and expressed as percentage of the unexposed control (mean ± S.D.). $^{*}p < 0.05$ as compared with vehicle control. $^{\#}p < 0.05$ as compared with CA alone.

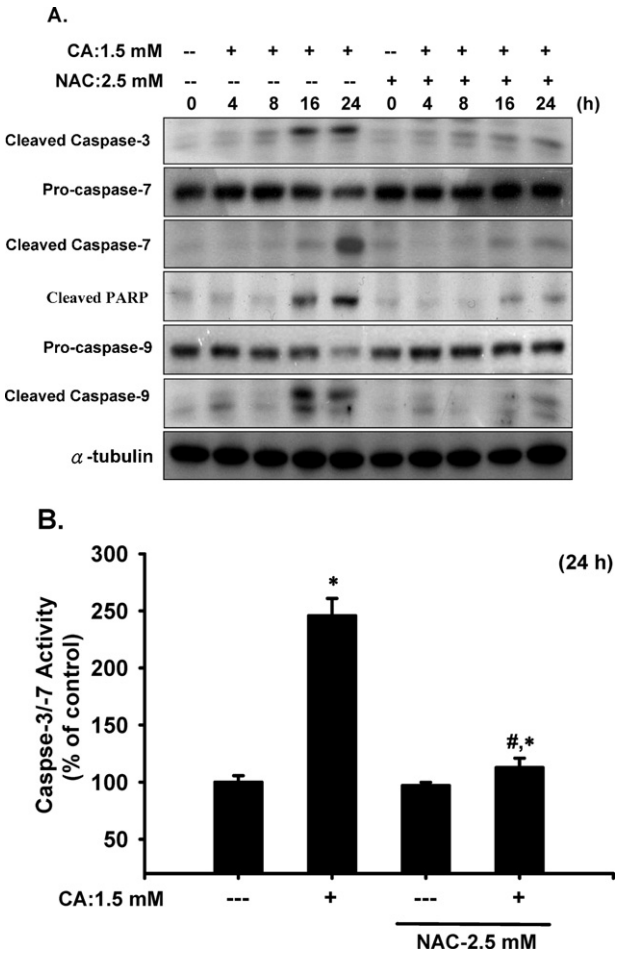

Fig. 4. Chloroacetic acid (CA) induced the expression of apoptosis-related proteins and caspase-3/-7 activity in Neuro-2a cells. Cells were treated with CA (1.5 mM) for different time intervals (4–24 h) in the absence or presence of NAC (2.5 mM). (A) The expressions of cleaved PARP and caspase-3, -7, and -9 pretein were examined by Western blot analysis. α-tubulin is used as an internal control, and representative images of three independent experiments are shown. (B) Caspase-3/-7 activity was measured by flow cytometry using fluorescent probe as described in Section 2. Data are presented as mean ± S.D. for four independent experiments with triplicate determination. $^{*}p < 0.05$ as compared with vehicle control. $^{\#}p < 0.05$ as compared with CA alone.

(Fig. 6D), and abrogated cytosolic cytochrome c release (Fig. 6E), MMP loss (Fig. 6F), and *Bcl-2* and *Bax* mRNA alterations (Fig. 6G). These results indicate that CA induces neuron cell death through ROS-mediated p38-MAPK activation regulated apoptotic signaling pathway.

4. Discussion

Chloroacetic acid is produced at a rate of more than 300,000 t annually and the widely used industrial chemical in the production of some herbicides and chemicals, in the intermediate for a number of synthetic products, and also the environmentally important contaminant (Reimann et al., 1996; Windholz, 1983). The disinfection of drinking water by chlorination processes is the most common practice in the United States and other countries of the world, thus the human may be at a constant risk of exposure to CA (one of major by-products) as disinfection by-products in finished drinking water or CA-contaminated food (significantly condensed during boiling or cooking) (Hua and Reckhow, 2007; Krasner et al., 1989; USEPA, 2002; Boorman, 1999). Moreover, CA has been found as an environmental contaminant of the atmosphere from

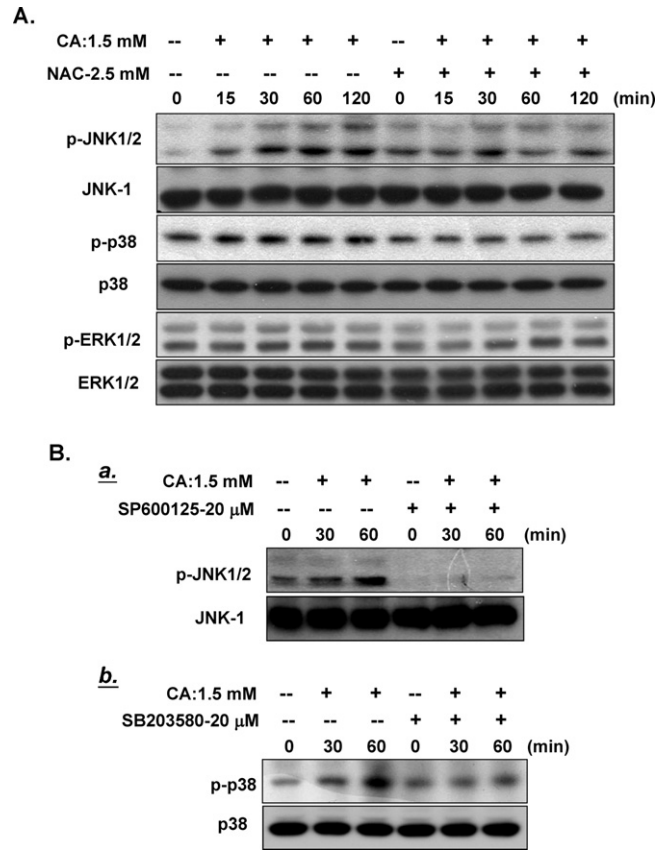

Fig. 5. Effects of chloroacetic acid (CA) on the activation of mitogen-activated protein kinase. Neuro-2a cells were treated with CA (1.5 mM) for 15–120 min in the absence or presence of NAC (2.5 mM), and the phosphorylation of JNK, p38-MAPK, and ERK1/2 proteins were examined by Western blot analysis (A). Furthermore, cells were pretreated with the specific JNK inhibitor (SP600125, 20 mM) or p38-MAPK inhibitor (SB203580, 20 mM) for 1 h and exposed to CA (1.5 mM). The levels of JNK and p38-MAPK protein phosphorylation were detected by Western bolt analysis (B, a and b, for 30 and 60 min). Results shown are representative of at least three independent experiments.

chlorinated hydrocarbons by photodechlorination reactions and as the metabolites of industrial compounds such as 1-chloroethene and 1,2-dichloroethane (Reimann et al., 1996; Rannug et al., 1976; Yllner, 1971). CA can be rapidly absorbed through the G-I tract and skin, highly corrosive to tissues, and lead to death following dermal exposures as well as after ingestion. It has been reported that CA induces variable degrees of injuries and histopathological changes in various organs of animal (Bhat and Ansari, 1989; Bhat et al., 1991; Dote et al., 2003) and causes acute and systemic toxicities in human by accidental or occupational exposure (Kulling et al., 1992; Kusch et al., 1990; Rogers, 1995). CA-inducing liver and kidney damages are accompanying by alterations of cytoskeletal, mitochondrial metabolic functions, and induction of cytotoxic responses (loss in cell viability, depletion of intracellular glutathione) *in vitro* (0.3–5 mM) and by increase in the levels of blood urea nitrogen, alanine aminotransferase, and aspartate aminotransferase *in vivo* (30–380 mg/kg) (Bhat et al., 1991; Bryant et al., 1992; Bruschi and Bull, 1993; Daniel et al., 1991; Dartsch et al., 2000). Furthermore, CA can penetrate and impair the function of the blood-brain barrier (BBB) into brain tissues, which causes injuries of the central nervous system, including loss of purkinje cells in the cerebellum and the production of pyknotic nuclei (Berardi et al., 1987; Bhat et al., 1990). The reports of human exposure to CA by accidental poisoning have indicated features of neurotoxic symptom occurred, including agitation, coma, disorientation, and cerebral edema, and plasma CA concentration are approximately 33–100 mg/L (0.35–1.1 mM) (Kulling et al., 1992; Pirson et al., 2003; Rogers, 1995). However, no antidote and the therapeutic model have been proven for CA intoxication, effectively. Moreover importantly, few studies are discussed with toxic effects of CA in nerve system, and the possible mechanisms underlying CA-induced neurotoxicity are mostly unclear. In the present study, the main findings showed that CA exposure significantly caused cell death, induced ROS generation (0.25–0.5 h) eliciting oxidative stress damage and the intracellular GSH depletion (8 h), which resulted in apoptotic expressions (including increase in sub-G1 hypodiploid population, the membrane externalization of PS, and caspase-3/-7 activity) in Neuro-2a cells. One of the mechanisms underlying CA-directed neuronal cell apoptosis was through mitochondrial dysfunction and downstream caspase cascades activation. These CA-induced cytotoxic responses could be effectively, but not full, reversed by pretreatment with antioxidant NAC. Our results indicate that oxidative stress-mediated mitochondria-dependent apoptotic signals are involved in CA-induced neuronal degeneration and cell death. Furthermore, the growing studies indicated that Fas/Fas ligand (FASL)/death receptor signaling- and endoplasmic reticulum (ER) stress-mediated cell death pathways are involved in the regulation of apoptosis in mammalian cells exposed to toxic chemicals (Daniel et al., 2003; Yen et al., 2012). It may be also different responses between the neuroblastoma cell lines and the primary cerebral granule neurons and/or the cerebral cortex of experimental animal, when that exposes to the same toxicant (LePage et al., 2005). However, the crucial role and detailed signals of these in CA-induced neuronal cell apoptosis still not clarify and require investigating in the future.

Oxidative stress can be elicited by ROS, which refers to persistent imbalance excessive ROS production and limited/destroy antioxidant defense, and has been implicated in a wide variety of biological responses such as apoptosis and nervous system injuries (Finkel and Holbrook, 2000). It has been indicated that there is causal relationship between the increase in ROS generation, which disrupts the pathophysiological functions of neural cells and causes apoptosis, and the progression of neurodegenerative disorders (Bharathi et al., 2006; Loh et al., 2006). Many studies have reported that toxic insults, such as chemicals and heavy metals, induced ROS generation causes the alteration of cellular function and the eventual result in neuronal cells injuries and apoptosis, which accompanies with triggering the activation of MAPKs (Dosunmu et al., 2007; Jomova et al., 2010; Kim et al., 2011; Mates et al., 2010; Xu et al., 2011). On the other hand, oxidative stress is a crucial role in mitochondrial dysfunction, which can further reduce ATP generation and exacerbate cell death signals. Mitochondria play a central role in both cell life and death by regulating the main energy production through TCA cycle activity and the associated electron transport chain, and deleterious ROS damage (Duchen, 2004; Pieczenik and Neustadt, 2007). Dysfunction of mitochondria, which induces by the production of excessive ROS, leads to disruption of MMP, release of cytochrome c and apoptosis inducing factor (AIF) (from the mitochondria space into cytosol), and then trigger to caspase cascades activation and apoptosis (Lu et al., 2011b; Susin et al., 1999; Tang et al., 2009). It has also been reported that mitochondrial dysfunction caused by mutation and reduction of mitochondrial DNA (mtDNA) and proteins, which are susceptible to oxidative stress, have been implicated in the pathogenesis of many diseases, including neurodegenerative diseases (Kang and Hamasaki, 2005; Kroemer and Reed, 2000). Furthermore, neuronal cells have been demonstrated to have low levels of antioxidant enzymes (such as catalase and glutathione peroxidase) to eliminate ROS; and brain, meanwhile, relies much on aerobic respiration and consumes the large quantity of oxygen leading it particularly vulnerable to oxidative stress (Coyle and Puttfarcken, 1993). Some studies showed that CA can induce lipid peroxidation and oxidative DNA damage, interfere with ATP formation in the tricarboxylic

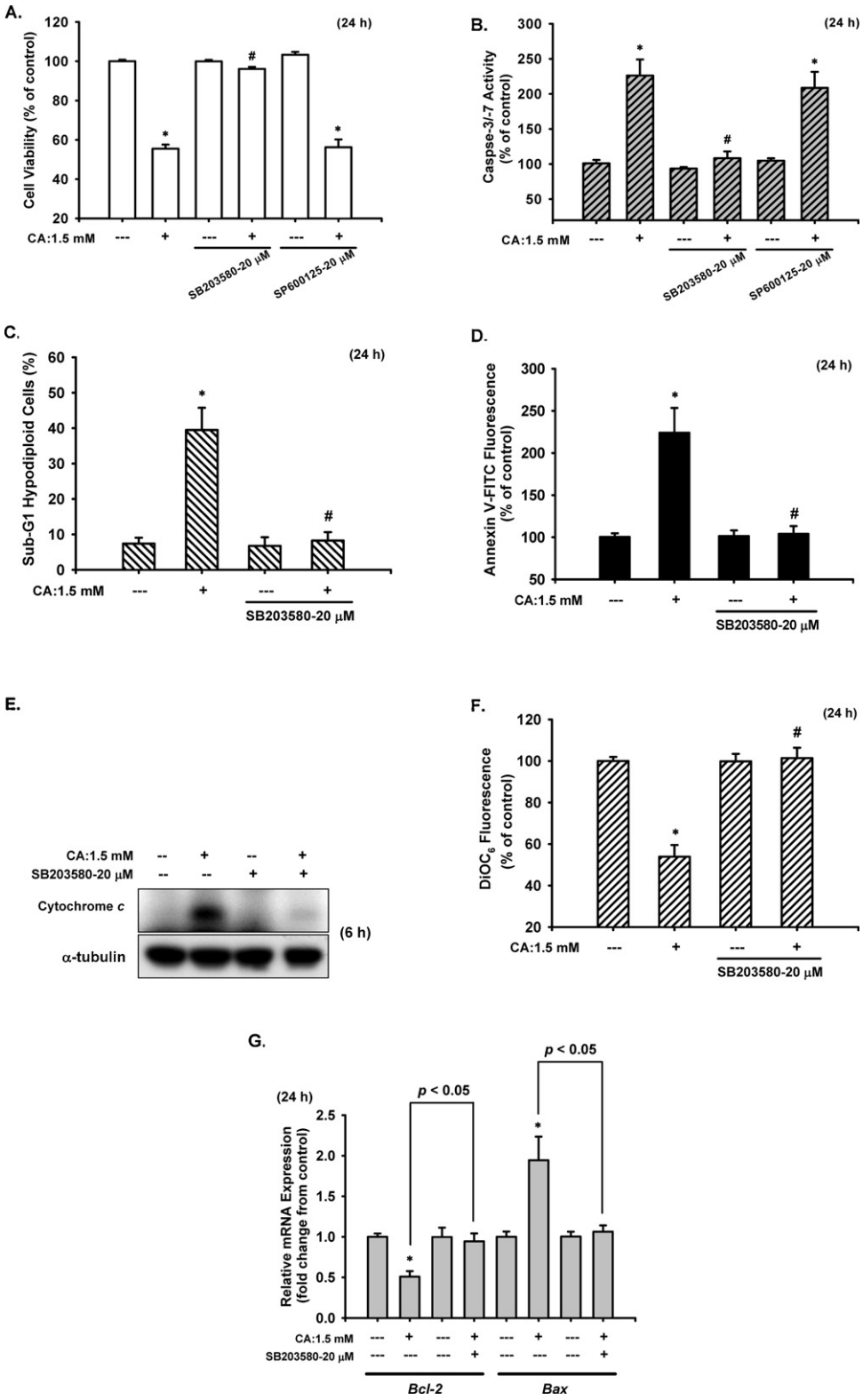

Fig. 6. Treatment of cells with p38-MAPK specific inhibitor prevent chloroacetic acid (CA)-induced apoptosis. Neuro-2a cells were treated with specific JNK inhibitor (SP600125, 20 mM) or p38-MAPK inhibitor (SB203580, 20 mM) for 1 h prior exposed to CA (1.5 mM). Cytotoxicity (cell viability) was determined by MTT assay (A, for 24 h); caspase-3/-7 activity (B, for 24 h), sub-G1 DNA content (C, for 24 h), annexin V-FITC fluorescence, and MMP depolarization (F, for 6 h) were analyzed by flow cytometry; cytosolic cytochrome c release was determined by Western blot analysis (E, for 6 h); and the expression of *Bcl-2* and *Bax* genes was analyzed by real-time quantitative RT-PCR (G, for 24 h) as described in Section 2. Data in A, B, C, D, F and G are presented as mean ± S.D. for four independent experiments with triplicate determination. $^{*}p < 0.05$ as compared with vehicle control. $^{\#}p < 0.05$ as compared with CA alone. Result shown in E is the representative image of at least three independent experiments.

acid (TAC) cycle and gluconeogenesis, and/or combine with the sulfhydryl group (reduce sulfhydryl content) in the mitochondria thereby destroying acetyl CoA-dependent biochemical processes and mitochondrial function leading to severe cell death and tissue damages such as liver, kidney, and central nervous system (Doedens and Ashmore, 1972; Kulling et al., 1992; Kupfer et al., 1994; Toshina et al., 2004); but, the precise mechanisms underlying CA-induced neuronal degeneration and cell death are not mostly understood, thus far. Here, our results work showed that CA was capable of causing apoptosis in Neuro-2a cells by inducing ROS generation and the intracellular GSH depletion in early stage (<8 h) and then triggering the MMP depolarization and cytochrome *c* release from the mitochondria into the cytosol and increasing in the activation of PARP, caspase-3, -7, and -9 proteases. In addition, our results also found the marked decrease in *Mcl-1* and *Bcl-2* expressions and increase in *Bax* expression in CA-treated Neuro-2a cells, which have been demonstrated the important regulators of mitochondrial-dependent apoptotic pathway (Leibowitz and Yu, 2010; Tang et al., 2009), and resulted in an increase in Bax/Bcl-2 ration leading to contribute to the promotion of CA-induced apoptosis. These CA-induced neuronal cell apoptotic responses could be effectively reversed by pretreated with NAC. Therefore, these results implicate that CA-caused neuronal cell death is through an oxidative stress-regulated mitochondrial-dependent apoptosis pathway.

MAPKs compose a family of protein kinases (including: JNK, ERK1/2, and p38-MAPK), which are major information transducers form the cell membrane to the nucleus, and control complex functions in mammalian such as differentiation, proliferation, and cell survival to death (Boutros et al., 2008; Pearson et al., 2001). MAPK activation has demonstrated to involve in apoptotic pathways and may play a crucial role in the pathogenesis of neurodegenerative disease (Kim and Choi, 2010; Schroeter et al., 2002). Recently, it has been pointed toward the MAPKs activation and oxidative stress trigger cellular damages as signals for many injurious agents toxicity to multiple cell types, particularly in neuronal cells (Kefaloyianni et al., 2006; Lu et al., 2011b; Son et al., 2011). The activation of JNK or p38-MAPK pathway, which is linked to oxidative stress, is been demonstrated the involvement in the progression of neuronal cell apoptosis (Choi et al., 2004; Marques et al., 2003). In addition, recent in vitro studies have shown that the activation of p38-MAPK-mediated mitochondria-dependent apoptosis can be induced by exposure to toxic insults (Kim et al., 2011; Lu et al., 2011a). The growing studies have also reported that the treatment with antioxidant-NAC can effectively abrogate the effects of toxic insults-induced ROS/p38-MAPK activation-regulated apoptosis pathways, indicating ROS-induced p38-MAPK activation play an important role in apoptosis (Lan et al., 2011; Lee et al., 2012; Lu et al., 2011b; Yen et al., 2011). However, to the best of our knowledge, there is no literature to investigate the role of MAPKs activation in CA-induced oxidative stress casusing neuronal cells death. The results of this study showed that CA dramatically enhanced the phosphorylation of JNK and p38-MAPK proteins, but not that of ERK1/2, in Neuro-2a cells. Pretreatment of cells with antioxidant NAC, pharmacological JNK inhibitor SP600125, and p38 inhibitor SB203580 suppressed CA-induced JNK and p38 MAPK activations. Only NAC and the specific p38-MAPK inhibitor effectively attenuated cytotoxicity, and reversed apoptosis events (including increase in caspase-3/-7 activity, sub-G1 hypodiploid population, and the membrane externalization of PS) and mitochondrial dysfunctions (including increase in cytochrome c release, MMP depolarization, and the changes of Bcl-2/Bax mRNA expression) in CA-treated neuro-2a cells. On the basis of these results, we indicate that p38-MAPK pathway activation plays the crucial role involved in CA-induced oxidative stress triggered neuronal cell apoptosis.

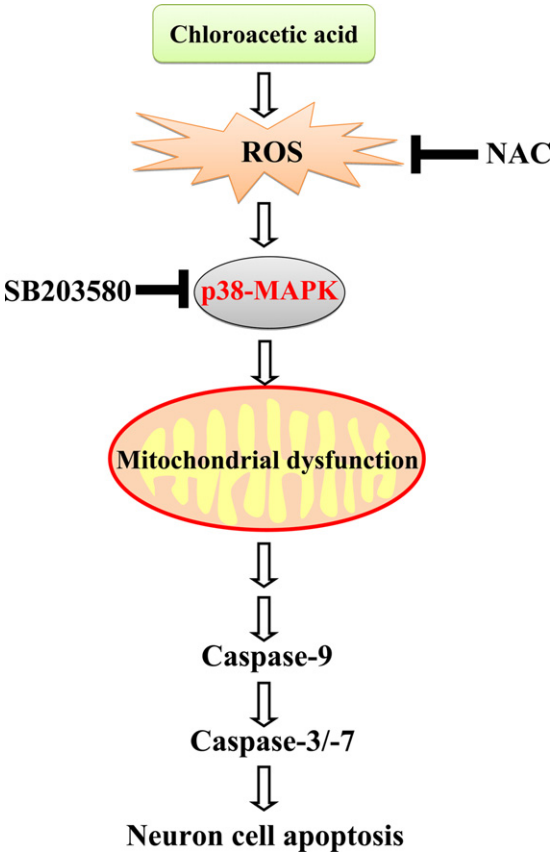

Fig. 7. Schematic diagram of the signal pathway involved in chloroacetic acid (CA)-induced apoptosis in neuronal cells. Proposed models show that CA causes neuronal cells apoptosis via ROS-mediated the activation of p38-MAPK pathway regulated mitochondria-dependent signaling cascades.

5. Conclusion

As indicated in Fig. 7, the results of this study provide evidence that CA is capable of inducing neuronal cell death, and more importantly reveal the underlying molecular basis of its effects. This present study has demonstrated that CA triggers cell death through which mitochondria dysfunction, which leads to activations of PARP and caspase cascades resulting in neuronal cells apoptosis. Furthermore, this adverse outcome can be prevented by antioxidant NAC and specific p38 inhibitor, suggesting that oxidative stress-induced p38-MAPK activated pathway plays an important role in CA-caused neuronal cell apoptosis. These finding are promising and warrant further study for the development of new therapeutic strategies for CA detoxication.

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Conflict of interest

All authors declare that they have no conflicts of interest related to this study.

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