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Carnosic acid, a rosemary phenolic compound, induces apoptosis through reactive oxygen species-mediated p38 activation in human neuroblastoma IMR-32 cells

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Running title: Carnosic acid induces apoptosis in neuroblastoma cells

Abbreviations CA, carnosic acid; JNK, c-Jun NH_2-terminal kinase ; ERK, extracellular signal-regulated kinase ; MAPK, mitogen-activated protein kinase; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide; NAC, *N*-acetylcysteine; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; ROS, reactive oxygen species.

Abstract Carnosic acid (CA), a rosemary phenolic compound, has been shown to display anti-cancer activity. We examined the apoptotic effect of CA in human neuroblastoma IMR-32 cells and elucidated the role of the reactive oxygen species (ROS) and mitogen-activated protein kinase (MAPK) associated with carcinogenesis. The result indicated that CA decreased the cell viability in a dose-dependent manner. Further investigation in IMR-32 cells revealed that cell apoptosis following CA treatment is the mechanism as confirmed by flow cytometry, hoechst 33258, and caspase-3/-9 and poly(ADP-ribose) polymerase (PARP) activation. Immunoblotting suggested a down-regulation of anti-apoptotic Bcl-2 protein in the CA-treated cells. In flow cytometric analysis, CA caused the generation of reactive oxygen species (ROS); however, pretreatment with the antioxidant *N*-acetylcysteine (NAC) attenuated the CA-induced generation of ROS and apoptosis. This effect was accompanied by increased activation of p38 and by decreased activation of extracellular signal-regulated kinase (ERK) as well as activation of c-Jun NH_2-terminal kinase (JNK). Moreover, NAC attenuated the CA-induced phosphorylation of p38. Silencing of p38 by siRNA gene knockdown reduced the CA-induced activation of caspase-3. In conclusion, ROS-mediated p38 MAPK activation plays a critical role in CA-induced apoptosis in IMR-32 cells.

Keywords Carnosic acid · apoptosis · reactive oxygen species · p38 kinase · human neuroblastoma IMR-32 cells

Introduction

Neuroblastoma, which is derived from cells of the sympathetic nervous system, is the most common solid extracranial neoplasm in children [1]. Neuroblastoma is a pediatric tumor that accounts for 15% of childhood cancer deaths and has a poor prognosis in children after 1 year of age [2]. Despite aggressive multimodal therapies, advanced neuroblastoma often acquires drug resistance and metastasizes [3]. Disruption of the apoptosis machinery plays an important role in the drug resistance of neuroblastomas. Many chemopreventive agents take effect by inducing apoptosis of neuroblastoma cells [4].

Apoptosis is characterized by morphological changes such as cell membrane blebbing, cell shrinkage, nuclear condensation, and formation of apoptotic bodies [5]. Activation of caspase is generally considered a hallmark of apoptotic cell death. The active caspase-9 recruits and activates procaspase-3, generating a fragment that activates the mitochondrial pathway. The DNA repair enzyme poly(ADP)-ribose polymerase (PARP) is shown to be cleaved by caspase-3 and as a result becomes incapable of responding to DNA damage during apoptosis [6, 7].

Recent studies have suggested that reactive oxygen species (ROS) may play an important role during apoptosis induction [8]. Many stimulants such as cigarette smoke, anticancer drugs, UV irradiation, and chemopreventive agents prompt cells to produce ROS. ROS induce a number of events in mediating apoptosis, including mitogen-activated protein kinases (MAPKs) signal transduction pathways [9]. Activated MAPKs play key roles in activating transcription factors and downstream kinases, leading to the induction of immediate-early gene expression and subsequent changes in other cellular processes [10]. The MAPKs are composed of several subfamilies, including the c-Jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinase. ERK and JNK are activated through receptor-mediated signaling stimuli and are associated with cell proliferation,

differentiation, and survival [11-13]. The p38 pathway is generally activated by stress agents and is implicated as a key regulator of stress-induced apoptosis in different cell types [14].

Rosemary (*Rosmarinus officinalis*), a commonly herb or spice, has been reported to possess a number of therapeutic applications in folk medicines. The rosemary phenolic compounds, in particular carnosic acid (CA) , carnosol, and rosemarinic acid, have some biological properties such as antiinflammatory, antioxidative, antiviral, and anticarcinogenic activities [15-18]. CA has been shown to inhibit lipid peroxidation [16] and to protect red cells against oxidative hemolysis [19]. Recently, interest has been growing in the anticarcinogenic properties of CA. Evidence has suggested that the arresting of human colonic adenocarcinoma Caco-2 cells in the G2/M phase by CA was shown to be caused by reduction of cyclin A [20]. In 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis model, the chemopreventive potential of CA is probably due to its modulating effect on carcinogen detoxification enzyme [21]. In addition, CA was shown to cause apoptosis and enhance the anticancer activity of vitamin D3 in HL-60 human leukemia cells [22, 23]. Moreover, the combined effect of CA and curcumin on apoptosis in acute myeloid leukemia cells is associated with activation of caspase-8, caspase-9, and caspase-3 and the proapoptotic protein Bid [24]. Although CA is considered to be an anti-cancer agent, its particular effects on neuroblastoma IMR-32 cells and the mechanisms involved remain unknown. In this study, we investigated the apoptosis effects of CA in human neuroblastoma IMR-32 cells. Moreover, we determined the involvement of ROS generation and the MAPK pathway in these processes.

Materials and methods

Chemical

Carnosic acid, leupeptin, aprotinin, Hoechst 33258 solution, paraformaldehyde, phosphatase inhibitor, HEPES, sodium bicarbonate, EDTA, glycerol, Triton X-100, dimethylsulfoxide (DMSO), sodium pyruvate, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rotenone, ascorbate, and *N*-acetylcysteine (NAC) were obtained from Sigma Chemical Company (St. Louis, MO). MEM medium, L-glutamine, nonessential amino acids, trypsin, sodium bicarbonate, and penicillin-streptomycin solution were obtained from Gibco Laboratory (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT).

Cell culture

Human neuroblastoma IMR-32 cells were purchased from Bioresources Collection and Research Center (BCRC, Taiwan). IMR-32 cells were grown in MEM medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 1×10^5 unit/L penicillin, 100 mg/L streptomycin, and 10% fetal bovine serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For all studies, cells between passages 3 and 10 were used. IMR-32 cells were plated on 35-mm plastic tissue culture dishes (Corning, NY) at a density of 0.7×10^6 cells per dish or on 60-mm plastic tissue culture dishes at a density of 2.5×10^6 cells per dish for Western blot analysis, and the dishes were treated until 70% confluence was reached. Cells were changed to fresh culture medium containing 2.5% fetal bovine serum for 12 h before CA treatment. Different concentrations of CA in 2.5% fetal bovine serum culture medium were then added, and the cells were incubated for the indicated times. Cells treated with 0.1% DMSO alone were regarded as controls. For antioxidant treatments, NAC at a concentration 2 mM and ascorbate

at a concentration 1 mM were added 1 h before CA treatment.

Cell viability

IMR-32 cells were plated on 35-mm plastic tissue culture dishes at a density of 0.7×10^6 cells per dish. Cell viability was determined by the MTT assay. Cells were stimulated with 5, 10, 20, 30, and 40 μM of CA for 24 h. MTT solution (5 mg/mL) was added to each dish and the dishes were incubated for 2 h. The formazan product was dissolved by the addition of 1 mL isopropanol to each dish with shaking for 10 min. Absorbance was detected at 570 nm by use of a microplate reader (Bio Rad, Japan).

Hoechst 33258 staining

IMR-32 cells were treated with 5, 10, 20, 30, and 40 μM CA for 24 h. After being washed with phosphate-buffered saline, the cells were fixed with 3.7% paraformaldehyde (pH 7.4) for 50 min. Subsequently, Hoechst 33258 nuclear dye was added to a final concentration 5 μg/mL for 1 h at 25°C in the dark. Morphological changes were observed by using a fluorescence microscope.

Annexin V and propidium iodide (PI) staining

IMR-32 cells were exposed to 0.1% DMSO or 30 μM CA for 12, 24, 36, 48, and 60 h. The Annexin V-FITC apoptosis detection kit (Becton Dickinson, San Diego, CA) was used according to the manufacturer's instructions. Following treatment, cells were harvested by trypsinization and washed with warm phosphate-buffered saline, centrifuged at 1,500 x *g* for 5 min at 25°C, and resuspended in 100 μl of 1X binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$]. Then Annexin-V FITC and PI were added for 15 min in the dark and finally 400 μL of 1X binding buffer was added. Samples were then immediately analyzed by use of a flow cytometer (Becton Dickinson, Heidelberg, Germany). Acquisition

gates of the cells and a minimum of 10,000 events were collected for each sample.

Western blot analysis

IMR-32 cells were washed with cold phosphate-buffered saline and were then harvested in lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 0.5 % Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and phosphatase inhibitor). Lysates were centrifuged at 14,000 x *g* for 20 min at 4°C. Protein concentrations were measured with a Coomassie plus protein assay reagent kit (Pierce, Rockford, IL). Thirty micrograms of protein from each sample was applied to 12.5% SDS-PAGE gels and was electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The nonspecific binding sites on the membranes were blocked at 4°C overnight with 50 g/L nonfat dry milk in 25 mM Tris/150 mM NaCl buffer, pH 7.4. The blots were then incubated with primary antibodies against procaspase-3, and -9 or cleaved caspase-3, and -9 or cleaved PARP (all purchased from Cell Signaling Technology, Beverly, MA); β-tubulin (purchased from Sigma Chemical Company, Louis, MO); JNK1, ERK1/2, phospho-JNK1, or phospho-ERK1/2 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA); p38 (purchased from Cell Signaling Technology, Beverly, MA); or phospho-p38 (purchased from Abcam, Cambridge, UK) overnight at 4°C and were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. The bands were detected by using an enhanced chemiluminescence kit (all purchased from Perkin Elmer Life Science, Boston, MA).

Measurement of ROS generation

Measurement of intracellular ROS production was made by using the peroxide-sensitive fluorescent probe 2,7-dichlorofluorescin diacetate (DCF-DA) (Molecular Probes Inc., Eugene, OR) as described previously [25]. In addition, mitochondrial was measured using MitoSOX

Red (Invitrogen, Carlsbad, CA). After reaching 90% confluence, cells were changed to fresh culture medium containing 2.5% fetal bovine serum for 12 h before CA treatment. Cells were changed to fresh culture medium containing 2.5% fetal bovine serum and 30 μM CA for 1, 3, 6, and 9 h. For examining the antioxidant effect, cells were pretreated with 2 mM NAC or 1mM ascorbate for 1 h and were then co-cultured with 30 μM CA for 6 h. An amount of 5 μM DCF-DA or 5 μM MitoSOX^{TM} red were then added to the medium for 45 min before the termination of CA treatment. DCF and MitoSOX Red fluorescence were measured in a flow cytometer (Becton Dickinson, Heidelberg, Germany).

Transient transfection of small RNA interference

IMR-32 cells were seeded at a density of 0.7 $\times 10^6$ cells/dish in a 35-mm plastic tissue culture dish. When 80% confluence was reached, for p38 small interfering RNA (siRNA) transfection, the cells were transfected with p38-siRNA (100 nM) or nontargeting control siRNA by using the DharmaFECT® siRNA transfection reagent according to the manufacturer's instruction (all from Thermo Fisher Scientific, Lafayette, CO) for 12 h. The sense sequences of these p38 siRNAs were as follows: 1) 5'-GGACCUCCUUAUAGACGAA-3', 2) 5'-GCACACUGAUGACGAAAUG-3', 3) 5'-ACACUCGGCUGACAUAAUC-3', and 4) 5'-GAAUGUGAUUGGUCUGUUG-3'. Twelve hours after transfection, the cells were changed to fresh culture medium containing 2.5% fetal bovine serum and 30 μM CA for 12 h or 24 h and protein expression was examined by Western blot analysis.

Statistical analysis

Statistical analysis was performed with commercially available software (SAS Institute Inc,Cary, NC). Data were analyzed by means of one-way ANOVA, and the significant difference among treatment means was assessed by use of Tukey's test. Differences between 2

groups were assessed using Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

CA inhibited cell viability in IMR-32 cells

The chemical structure of CA is shown in Fig. 1. First, we investigated the effect of CA treatment on the viability of human neuroblastoma IMR-32 cells. In cells exposed to 5, 10, 20, 30, and 40 μM CA for 24 h, cell viability was reduced in a dose-dependent manner (P <0.05) (Fig. 2). CA exhibited potent cytotoxic activity against neuroblastoma IMR-32 cells, with an IC_{50} value of approximately 30 μM.

Effect of CA on cell morphology of IMR-32 cells

To determine whether the reduced cell viability was due to apoptosis, IMR-32 cells were stained with Hoechst 33258. In the control group, the IMR-32 cells were homogeneously stained (Fig. 3). Nuclear condensation and fragmentation were significantly increased in the cells treated with 30 and 40 μM CA for 24 h. In addition, cells treated with CA were shown to have apoptotic bodies by phase-contrast microscopy.

CA induced apoptosis in IMR-32 cells

To further confirm the apoptosis, the cells were examined by flow cytometric analysis using double staining of Annexin V-FITC and PI. As shown in Fig. 4, the apoptotic cells were observed in IMR-32 cells treated with CA for 24 h. In the cells treated with CA, the apoptotic population increased gradually throughout the culture period. CA at 60 h increased the apoptotic population by 3.5-fold compared with that of the control cells.

CA induced the expression of apoptosis regulatory proteins

To clarify the mechanism of CA-induced apoptosis, we examined changes in the caspase family proteins and anti-apoptotic protein Bcl-2 by Western blot analysis. CA significantly

reduced procaspase-9 and -3 but markedly increased the cleaved forms of caspase-9, -3, and PARP in a dose-dependent manner (Fig. 5). The ratio of cleaved to procaspase-9 and -3 was increased in the CA-treated group (P <0.05). However, caspase-8 protein was not expressed after cells were treated with CA (data not shown). The level of anti-apoptotic Bcl-2 protein was reduced in cells treated with 30 and 40 μM CA. These results suggested that the induction of cell death by CA mainly involved activation of the apoptotic mitochondrial pathway.

Generation of ROS in CA-induced apoptosis

We next explored whether ROS generation was involved in the CA-induced apoptosis of IMR-32 cells. The results of the flow cytometry analysis using DCF-DA as a fluorescent ROS indicator showed that intracellular ROS level was gradually increased and reached a maximum at 6 h and then decreased in the presence of CA (Fig. 6A). Compared with the control group, there was a 1.6-fold increase at 6 h. Pretreatment with NAC reduced CA-induced ROS generation by 39%. In addition, we further used the mitochondrial targeted ROS probe-MitoSOX Red to confirm the ROS production. Increases of 2.3 and 2.5 fold in MitoSOX Red fluorescence intensity were noted in cells cultured with CA and rotenone (a mitochondrial inhibitor), respectively, as compared with the control cells. Pretreatment with ascorbate reduced CA-induced mitochondrial ROS generation by 21% (Fig. 6 B). Immunoblots also revealed that CA induced the cleavage of caspase-9, caspase-3, and PARP protein and reduced procaspase-9 and -3 proteins (Fig. 6 C). In contrast, both NAC and ascorbate decreased the CA-induced cleavage of caspase-9, caspase-3, and PARP protein (Fig. 6 C). These findings suggested that the generation of ROS may play an important role in the CA-induced apoptosis in IMR-32 cells.

Role of MAPKs in CA-mediated apoptosis

Activation of the MAPK cascades is considered to play a crucial role as a regulator of apoptotic signaling pathways [9]. Therefore, we attempted to determine whether CA-induced apoptosis was regulated by JNK1, ERK, and p38 kinase. As shown in Fig. 7 A, the activation of p38 was notably increased after the cells were treated with CA for 12 and 24 h, and returned to basal level after 36 and 48 h. However, the phosphorylation of ERK and JNK1 was decreased in a time-dependent manner (Fig. 7 B). Cells pretreatment with NAC attenuated the activation of p38 by CA, and had little effect on the phosphorylation of ERK and JNK1. These results suggested that the activation of p38 was mainly involved in the CA-induced ROS generation.

To confirm the involvement of p38 activation in the CA-induced apoptosis, we used knockdown of p38 by siRNA transfection. Immunoblots revealed that CA increased the activation of p38 and caspase-3 (Fig. 8 A). With p38 siRNA, the cellular p38 level was decreased (vs. si-control), which resulted in alleviation of the phosphorylation of p38 by CA. The activation of caspase-3 expression by CA was then suppressed (Fig. 8 B).

Discussion

Rosemary extracts have been widely investigated for their antiproliferative and anticarcinogenic properties [26, 27]. Application of rosemary extracts was shown to prevent DNA damage and tumor formation by 7,12-dimethylbenz[*a*]anthracene in mouse skin and rat mammary gland [26, 28]. The accumulated evidence supports that rosemary extracts inhibit benzo[*a*]pyrene-induced genotoxicity in human bronchial cells, and the components of rosemary, such as CA, carnosol, and rosmarinic acid, were responsible for this effect [29]. Carnosol displays growth-inhibitory effects in human prostate cancer PC3 cells by G2-phase cell cycle arrest [17]. Rosmarinic acid induces apoptosis and inhibits the proliferation of human HCT115 colorectal cells via MAPK/ERK pathway [30]. In human colon adenocarcinoma COLO 205 cells, the rosmanol extracted from rosemary is capable of inducing apoptosis through both a mitochondria-mediated pathway and a receptor-mediated pathway [31]. Recent studies have suggested that CA inhibits the proliferation of Caco-2 cells by causing cell cycle arrest at the G2/M phase and induces apoptosis in human promyelocytic leukemia HL-60 cells [20, 23]. Moreover, the combinatorial effect of CA and curcumin on apoptosis in acute myeloid leukemia cells was associated with activation of caspase-9 and caspase-3 and the pro-apoptotic protein Bid [24]. The results of the present study suggest that CA induced the apoptosis of IMR32 neuroblastoma cells via the mitochondrial pathway. We suggest that the generation of ROS by CA leads to activation of the p38 pathway, which results in apoptosis.

Caspases are a family of cysteine proteases that play a central role during the executional phase of apoptosis. Several chemotherapeutic drugs induce cell death through the caspase-mediated apoptosis pathways. Activation of caspase-8 is via the extrinsic apoptosis pathway, which is induced by triggering of the death receptors pathway. Our results indicated that caspase-8 protein was not expressed after cells were treated with CA for 24 h (data not

shown). This finding is supported by the findings of others that some neuroblastoma cell lines such as IMR-32 do not express caspase-8 protein [32]. Moreover, loss of caspase-8 expression has been reported in patients with highly malignant neuroblastoma [33]. In the intrinsic apoptosis pathway, upon apoptotic stimulation, initiator caspases such as caspase-9 are cleaved and activated. The activated upstream caspases further process the downstream executioner caspases, such as caspase-3 and -7, by cleaving them into large and small subunits, thereby initiating a caspase cascade leading to apoptosis [7]. The Bcl-2 family proteins, the pro-apoptotic Bax and the anti-apoptotic Bcl-2, regulate cell death by controlling mitochondrial membrane permeability during apoptosis [34]. A decrease in the levels of Bcl-2 leads to the loss of mitochondrial transmembrane potential, a key event in the induction of apoptosis, and opens mitochondrial permeability transition pores [35]. Isobavachalcone, a chalcone constituent of *Angelica keiskei*, induces apoptotic cell death with caspase-3 and -9 activation and Bax upregulation in neuroblastoma IMR-32 and NB-39 cells [32]. Zn deficiency triggers IMR-32 apoptotic death associated with the intrinsic pathway, which can be a consequence of ERK inhibition and caspase-3 activation [36]. However, xanthoangelol, another chalcone constituent of *Angelica keiskei*, induces apoptotic cell death by activation of caspase-3 in neuroblastoma IMR-32 cells through a mechanism that does not involve Bax/Bcl-2 signal transduction [37]. In the present study, both CA and rotenone induced apoptotic cell death with Bcl-2 downregulation and caspase-9 and caspase-3 activation, resulting in cleavage of PARP in IMR32 neuroblastoma cells (Fig. 5). Taken together, these results indicate that the CA-induced cell death involved activation of the apoptotic mitochondrial pathway.

Recent studies have indicated that cancer chemopreventive agents induce apoptosis in part by the generation of ROS and the disruption of redox homeostasis [38]. The generation of ROS induces mitochondrial cytochrome *c* release, in which sequential activation of caspase-9

and -3 occurs [39]. The induction of apoptosis by garlic diallyl disulfide is associated with the production of ROS and activation of caspase-3 in Ca Ski cells [40]. In addition, surfactin induces apoptosis in human breast cancer MCF-7 cells through a ROS-mediated mitochondrial/caspase pathway [41]. In the present study, we monitored the change in cellular redox status by the DCF-DA and MitoSOX Red cytofluorimetric assay. With CA treatment, ROS production gradually increased and reached a maximum at 6 h and then decreased (Fig. 6 A) Moreover, this effect was reduced by pretreatment with NAC and ascorbate. This suggests that the activation of the apoptosis caspase cascade can be explained, at least in part, by a change in redox states caused by CA (Fig. 6 C).

MAPKs control many cellular events, including differentiation, proliferation, and apoptosis [12, 14, 42]. JNK regulates serotonin-mediated proliferation and migration in pulmonary artery smooth muscle cells [12]. Treatment of IMR-32 cells with CdSe-core induces mitochondrial-dependent apoptotic processes by inhibiting ERK survival signaling [13]. Xavier and co-workers presented that romarinic acid induces apoptosis and inhibits the proliferation of human HCT115 colorectal cells via inhibition of the ERK pathway [30]. In particular, p38 is known to play a critical role in the transmission of apoptotic signals [43]. Indole ethyl isothiocyanate is thought to inhibit the cell proliferation and cell viability of neuroblastoma SMS-KCNR through activation of p38 signaling [14]. The p38 MAPK pathway is also critical for 5,5'-dibromodiindolylmethane-induced apoptosis to prevent oral squamous carcinoma cells [42]. These findings agree with our results that IMR-32 cells treated with CA activated p38 protein and down-regulated ERK1/2 and JNK protein (Fig. 7A). Furthermore, the CA-induced activation of p38 through a ROS-dependent mechanism was evidenced by inhibition of p38 phosphorylation by NAC (Fig. 7 B). Pretreatment with p38 siRNA attenuated the activation of p38 and caspase-3 by CA (Fig. 8 A and 8 B). These data suggest that the p38 pathway played an important role in the generation of ROS by CA, which

induced apoptosis of IMR-32 cells. This explanation is similar by the finding that the activation of the p38 signaling pathway by arachidonic acid and the resulting induction of human leukemia U937 cell apoptosis are prevented by NAC [38].

In conclusion, the results of the present study indicate that CA induces apoptotic cell death though the mitochondrial pathway in human neuroblastoma IMR-32 cells. Moreover, ROS-mediated phosphorylation of p38 could play a critical role in CA-induced apoptosis.

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Fig. 1 Chemical structure of carnosic acid (CA).

Fig. 2 Carnosic acid (CA) inhibited cell growth in human neuroblastoma IMR-32 cells. IMR-32 cells were treated with 0.1% dimethylsulfoxide (DMSO) alone (control, -) or with 10, 20, 30, or 40 μM of CA for 24 h. Cell viability was assessed by using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The level in the control cells was set at 100%. Values are shown as the means±SD of four independent experiments. Means without a common letter differ, $P < 0.05$.

Fig. 3 Carnosic acid (CA) induces nuclear morphology changes in human neuroblastoma IMR-32 cells. Nuclei were visualized with Hoechst 33258 staining. Cells were treated with 0.1% dimethylsulfoxide (DMSO) alone (control, -) or with 5, 10, 20, 30, or 40 μM of CA for 24 h. Upper panels show the phase contrast image and lower panels show the fluorescent image. Phase contrast and fluorescent images were obtained from the same view (magnification, 200 x). Arrows indicate apoptotic cells. One representative image out of four independent experiments is shown.

Fig. 4 Carnosic acid (CA) induces apoptosis in human neuroblastoma IMR-32 cells. Cells were exposed to the medium with 0.1% dimethylsulfoxide (DMSO) alone (control, -) or with 30 μM CA for 12, 24, 36, 48, and 60 h. Cell distribution was analyzed by using Annexin V-FITC binding and propidium iodide (PI) uptake as described in the Materials and Methods. FITC and PI fluorescence were measured by flow cytometry. In these dot graphs, Q1-1 indicates necrotic cells (Annexin V^-/PI^+), Q2-1 indicates late apoptotic cells (Annexin V^+/PI^+), Q3-1 indicates viable cells (Annexin V^-/PI^-), and Q4-1 indicates early apoptotic cells

(Annexin V^+/PI^-). Bars are the percentage of early and late apoptotic cells. Values are expressed as the means±SD of three representative experiments. Groups not sharing a common letter differ significantly, *P <0.05*.

Fig. 5 Carnosic acid (CA) dose-dependently increased apoptotic regulatory proteins in human neuroblastoma IMR-32 cells. Cells were treated with 0.1% dimethylsulfoxide (DMSO) alone (control, -) or with 10, 20, 30, or 40 μM CA for 24 h to determine the protein levels. The cleaved caspase/procaspase ratio relative to the control group (mean±SD) is shown. Normalization of Western blots was ensured by β-tubulin. The level in control cells was regarded as 1. Means without a common letter differ, *P* <0.05. One representative immunoblot out of four independent experiments is shown.

Fig. 6 Carnosic acid (CA)-induced apoptosis is associated with the generation of intracellular reactive oxygen species (ROS) in human neuroblastoma IMR-32 cells. Cells were cultured with 0.1% dimethylsulfoxide (DMSO) alone (control, -) or with 30 μM of CA for 1, 3, 6, and 9 h, or with 50μM of rotenone for 6 h . For examining the antioxidant effect, cells were pretreated with 2 mM NAC or 1 mM ascrobate for 1 h and then co-cultured with CA for 6 h. (a) DCF fluorescence and (b) MitoSOX Red fluorescence were measured by flow cytometry. The level in the control cells was set at 1. Values are shown as the means±SD of four independent experiements. Means without a common letter differ significantly, *P* <0.05. *Different from CA or rotenone alone in control group, *P* <0.05. #Different from CA co-cultured with ascrobate in CA alone group, *P* <0.05. (c) The expression of indicated proteins was analyzed by Western blotting. One representative immunoblot out of four independent experiments is shown.

Fig. 7 Effect of carnosic acid (CA) on the activation of ERK1/2, JNK1, and p38 in human neuroblastoma IMR-32 cells. Cells were cultured with 0.1% dimethylsulfoxide (DMSO) alone (control, -) or with 30 μM of CA for 12, 24, 36, and 48 h. (a) Activation of ERK1/2, JNK1, and p38 was assessed by immunoblot analysis of the phosphorylated forms (P-) of the mitogen-activated protein kinases in whole cell lysates. β-Tubulin was used as the loading control. (b) The expression of indicated proteins was analyzed after incubation with CA for 12 h in the presence or absence of NAC, which was added to cells 1 h before CA treatment. One representative immunoblot out of three independent experiments is shown.

Fig. 8 Carnosic acid (CA)-induced activation of caspase-3 was inhibited by p38-siRNA in human neuroblastoma IMR-32 cells. Cells were transfected with p38-siRNA (si-p38) or nontargeting control siRNA (si-control) for 12 h. The transfected cells were then treated with 30 μM of CA for 12 and 24 h. The activation of p38 and capase-3 were measured by Western blotting. β-tubulin was used as the loading control. One representative immunoblot out of three independent experiments is shown.

Fig. 1

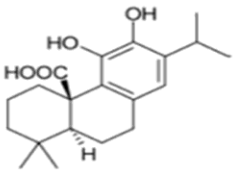
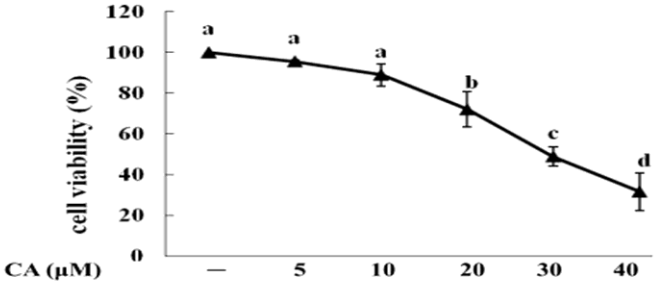


Fig. 2



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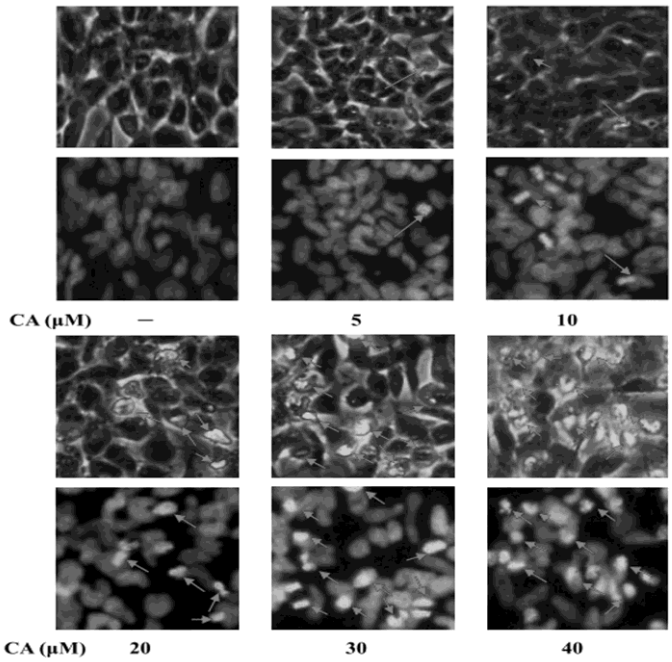

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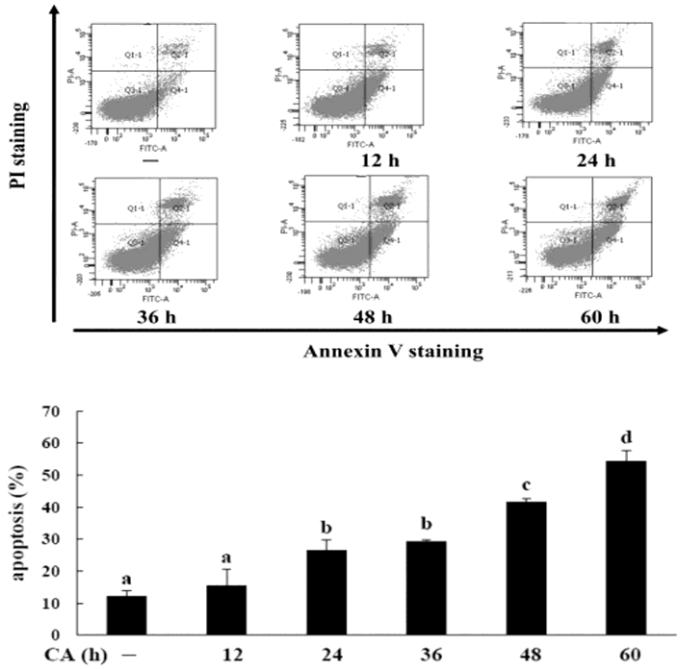

PI staining
—
12 h
24 h
36 h
48 h
60 h
Annexin V staining
apoptosis (%)
CA (h) —
12
24
36
48
60

Fig. 5

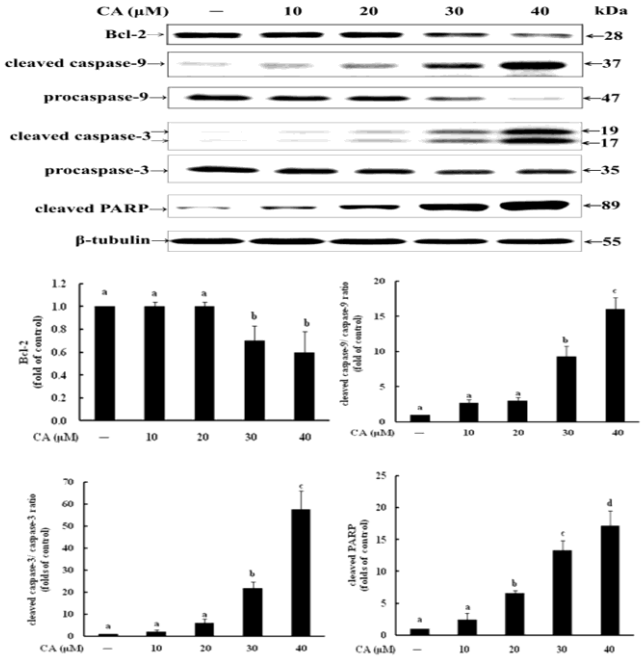

CA (μM)
—
10
20
30
40
kDa
Bcl-2
cleaved caspase-9
procaspase-9
cleaved caspase-3
procaspase-3
cleaved PARP
β-tubulin
28
37
47
19
17
35
89
55
Bcl-2 (fold of control)
cleaved caspase-9/ caspase-9 ratio (fold of control)
cleaved caspase-3/ caspase-3 ratio (fold of control)
cleaved PARP (fold of control)

Fig. 6

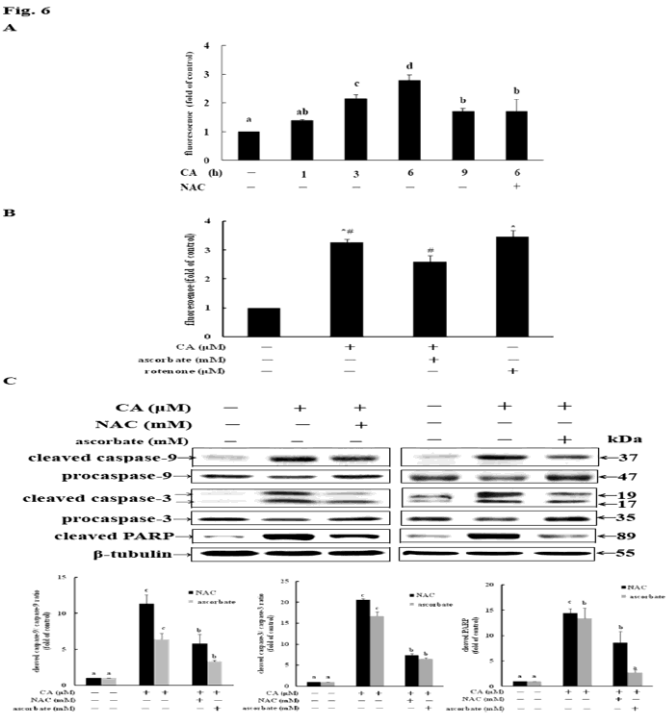

A
B
C
CA (μM)
NAC (mM)
ascorbate (mM)
rotenone (μM)
cleaved caspase-9
procaspase-9
cleaved caspase-3
procaspase-3
cleaved PARP
β-tubulin
kDa
37
47
19
17
35
89
55

Fig. 7

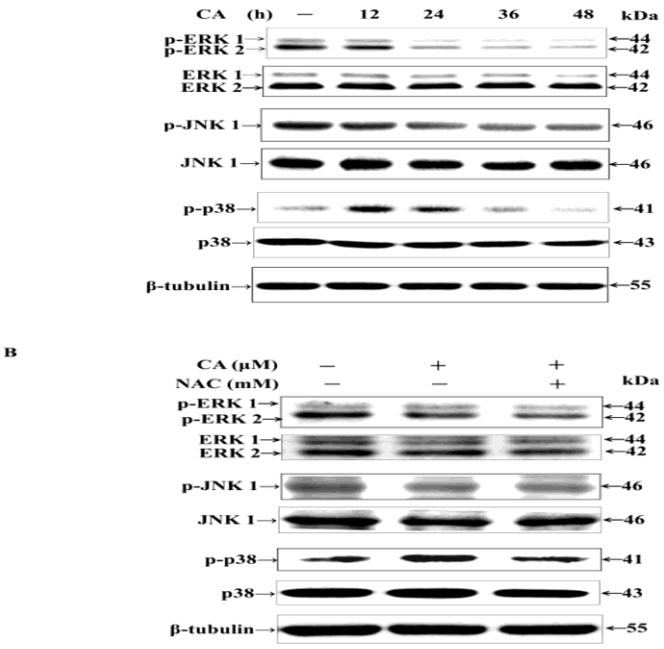
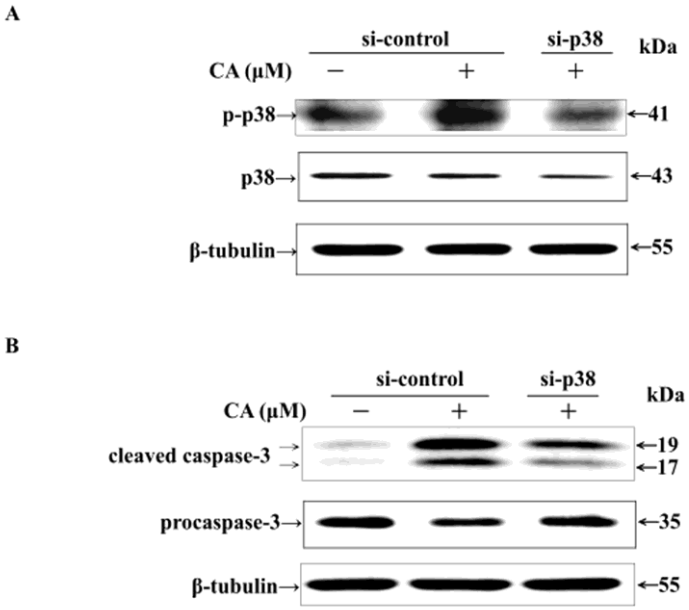

A
B
CA (h)
CA (μM)
NAC (mM)
p-ERK 1
p-ERK 2
ERK 1
ERK 2
p-JNK 1
JNK 1
p-p38
p38
β-tubulin
kDa
44
42
46
41
43
55

Fig. 8


A
si-control
si-p38
kDa
CA (μM)
−
+
+
p-p38→
←41
p38→
←43
β-tubulin→
←55
B
si-control
si-p38
kDa
CA (μM)
−
+
+
cleaved caspase-3
←19
←17
procaspase-3→
←35
β-tubulin→
←55