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Induction of apoptotic cell death by 2′-hydroxycinnamaldehyde is involved with ERK-dependent inactivation of NF-κB in TNF-α-treated SW620 colon cancer cells

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Abstract

2′-Hydroxycinnamaldehyde (HCA) inhibits cell growth of several human cancer cells with unknown mechanisms. We investigated the inhibitory effect of HCA on TNF-α-induced cell growth and possible signal pathway in SW620 colon cancer cells. HCA inhibited TNF-α-induced SW620 colon cell growth in time- and dose-dependent manner through induction of apoptotic cell death. Parallel with inhibitory effect on cell growth, HCA dose dependency inhibited TNF-α-induced activation of NF-κB accompanied with inhibition of the translocation of p50. HCA also induced expression of caspase-3 and Bax, but decreased Bcl-2. HCA furthermore activated ERK pathway, and ERK inhibitor reversed inhibitory effect of HCA on cell growth and transcriptional activation of NF-κB. These results demonstrate that HCA inhibits cell growth through induction of apoptotic cell death by ERK pathway-dependent NF-κB inactivation.

Keywords: 2′-Hydroxycinnamaldehyde; NF-κB; ERK; Apoptotic cell death; Colon cancer

1. Introduction

Nuclear factor of κB (NF-κB) is known to be involved in the inflammatory and innate immune responses [1]. Although the importance of NF-κB in immunity is undisputed, recent evidence indicates that NF-κB activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis, proliferation, differentiation, and migration of the cells. Activation of NF-κB has been found in tumor promotion and progression of skin cancers [2]. NF-κB activation is also associated with colorectal cancer development. Colon cancer cell lines and human tumor samples as well as nucleic of stromal macropharges in sporadic adenomatous polyps were found to have increased NF-κB activity [3,4]. Exposure to environmental carcinogens can activate oncogene or can lose tumor-suppressor gene activity through constitutive NF-κB activation [1]. NF-κB can lead to further proliferate transformed cells through enhanced production of growth factors and cytokines [1]. Inactivation of NF-κB in many cancer cells by chemotherapy or by radiation has been demonstrated to blunt the ability of the cancer cells to growth [5].

In TNF signaling pathway, NF-κB act as a cell survival mechanism through its regulatory role over the expression of an array of anti-apoptotic genes, including Bcl-2 family proteins, Mn-superoxide dismutase, and cyclooxygenase-2 [6,7]. Treatment of SW620 colon cells with sulfasalazine, an anti-inflammatory agent inhibited TNF-α, LPS, or phorbol ester-induced NF-κB activation [8]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin have been shown to suppress transcription factor NF-κB, which controls the expression of genes such as cyclooxygenase (COX)-2 and cyclin D1, leading to inhibition of proliferation

Abbreviations: HCA, 2′-hydroxycinnamaldehyde; NF-κB, nuclear transcription factor-κB; TNF-α, tumor necrosis factor-α; ERK, intracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; IKK, IκB kinase; EMSA, electro mobility shift assay; JNK, c-jun NH_2-terminal kinase

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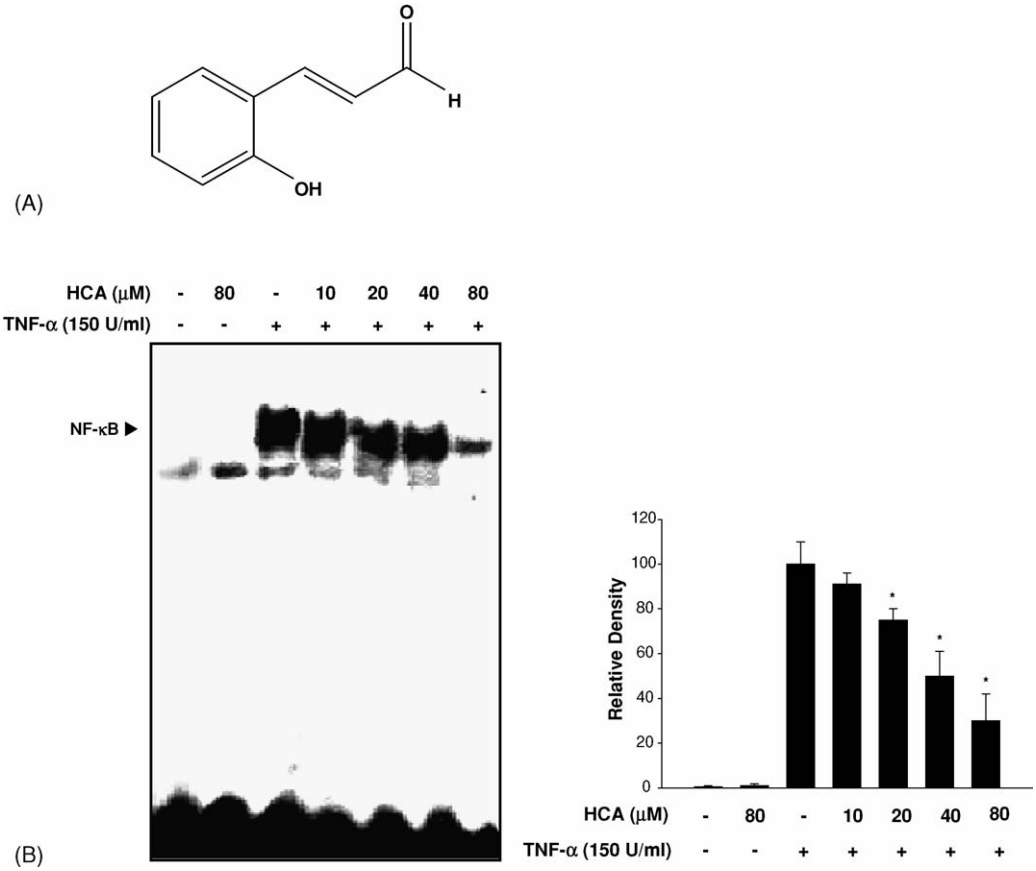

Fig. 1. Effect of HCA on TNF-α-induced NF-κB activation in SW620 cells. (A) Structure of 2′-hydroxycinnamaldehyde (3-2′-hydroxyphenyl)-2-propenal); (B) the activation of NF-κB was investigated using EMSA as described in Section 2. Nuclear extract from SW620 cells treated with TNF-α alone (150 U/ml) or the combination with HCA (10, 20, 40 and 80 μM) was incubated in binding reactions of ^{32}P-end-labeled oligonucleotide containing the κB sequence. NF-κB DNA-binding activity was determined by EMSA. The density ratios of NF-κB DNA-binding were calculated, and the mean value in the untreated control group was set to 100. All values represent means ± S.E. of three independent experiments performed in triplicate. $^{*}P < 0.05$ indicates statistically significant differences from the TNF-α-treated group.

of tumor cells [8–10]. Other NSAIDs (ibuprofen, sulindac, phenylbutazone, naproxen, indomethacin, diclofenac, dexamethasone, celecoxib, and tamoxifen) and natural products (resveratrol, curcumin) have been also demonstrated to inhibit NF-κB activity resulting in the inhibition of cancer cell growth through induction apoptosis [11].

The signaling event implicated in survival, growth arrest, or programmed cell death includes the activation of mitogen-activated protein kinase (MAPK) pathway [12]. ERK activation plays an active role in mediating chemotherapy agents such as retinoids, cisplatin-induced apoptotic cell death signal [13–15]. Accumulating evidence indicates that NF-κB activation is modulated by MAPK family (ERK, JNK as well as p38 MAPK) [16,17]. Therefore, modulation of these signal proteins could be related to control of cancer cell growth.

2′-Hydroxycinnamaldehyde (HCA), a derivative of cinnamaldehydes, isolated from the stem bark of *Cinnamomum cassia* (Fig. 1), has been reported to inhibit farnesyl protein transferase activity in vitro [18], angiogenic activity [19], and immunomodulating activity [20]. HCA has been also shown to inhibit proliferation of several human cancer cell including breast, leukemia, ovarian, lung, and colon cancer cells [21]. However, little is known about the putative mechanism of growth inhibitory effect of HCA. HCA has an α,β-unsaturated carbonyl group, which could react with nucleophiles, especially cysteine sufhydryl groups of molecules, by a Michael type addition [22,23]. Therefore, exposed thiol groups, such as cysteine residues in proteins, were appearing to be the primary targets of HCA. Since NF-κB has p50 and p65 subunits containing cysteine residues in their DNA-binding domains [24–26], NF-κB can be targeted by HCA. We previously found that HCA inhibited LPS-induced NF-κB activity in RAW 264.7 cells [27]. Consideration of the importance of NF-κB in cancer cell growth, we investigated whether HCA can inhibit cell growth through inactivation of NF-κB, and further investigated possible related mechanisms in SW620 human colon cells.

2. Materials and methods

2.1. Chemicals

2′-Hydroxycinnamaldehyde was isolated from the stem bark of *Cinnamomum cassia* Blume and synthesized from

2-hydroxycinnamyl alcohols according to the reported method [18]. HCA was dissolved in dimethyl sulfoxide (DMSO) and used at the concentrations indicated. TNF-α and PD98059 were purchased from Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise mentioned.

2.2. Cell culture

SW620 human colon cells were obtained from the American Type Culture Collection (Cryosite, Lane Cove NSW, Australia). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum were purchased from Gibco Life Technologies (Rockville, MD, USA). SW620 human colon cells were grown in RPMI1640 with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2 humidified air.

2.3. Cell viability assay

To determine the appropriate dose that is not cytotoxic to the cells, the cytotoxic effect was evaluated in the cells cultured for 24, 48 and 72 h using the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide MTT assay [28]. Briefly, the cells plated on 96-well plates at a concentration of 1.5×10^3 cells/cm^2. The cells were incubated at 37 °C in 5% CO_2, and were treated with TNF-α (150 U/ml) and HCA (0, 10, 20, 40 and 80 μM). After the incubation for 12, 24, 36 or 72 h, the cells were washed twice with 1× PBS, followed by the addition of 1 ml of PBS, MTT was dissolved in without phenol red at a concentration of 10 μg/ml, 10 μl of this solution was then added to cell cultured for designed time. After 4 h, cultures were removed from the incubator and the formazan crystals dissolved by adding 100 μl solubilization solution (0.04N HCl in isopropanol). Metabolic activity was quantified by measuring light absorbance at 570 nm.

2.4. Western blot analysis

Cultured cells were washed twice with 1× PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM PMFS, 10 μl/ml aprotinin, 1% igapel 630 (Sigma Chemical Co., St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate], and centrifuged at 23,000 × *g* for 1 h. The protein concentration was measured by the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories Inc., Hercules, CA, USA), and equal amount of proteins (20 μg) were separated on a SDS/12%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% Tween-20. The membrane was incubated for 5 h at room temperature with specific antibodies: rabbit polyclonal antibodies against p65 (1:500) and goat polyclonal for p50 (1:500), and mouse monoclonal ERK antibody (1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the ECL Western blotting detection system. The relative density of the protein bands was scanned by densitometry using Mylmage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, CA, USA).

2.5. Gel electromobility shift assay

Gel shift assays were performed according to the manufacturer's recommendations (Promega, Madison, Wl, USA). Briefly, 1×10^6 cells/ml was washed twice with 1× PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were spun down at 15,000 × *g* for 1 min, and the resulting supernatant was removed. Solution A (50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 0.5% Nonidet P-40) was added to the pellet in a 2:1 ratio (v/v) and allowed to incubate on ice for 10 min. Solution C (solution A + 10% glycerol and 400 mM KCl) was added to the pellet in a 2:1 ratio (v/v), and vortexes on ice for 20 min. The cells were centrifuged at 15,000 × g for 7 min, and the resulting nuclear extract supernatant was collected in a chilled Eppendorf tube. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP for 10 min at 37 °C. Gel shift reactions were assembled and allowed to incubate at room temperature for 10 min followed by the addition of 1 μl (50,000–200,000 cpm) of ^{32}P-labeled oligonucleotide and another 20 min of incubation at room temperature. Subsequently, 1 μl of gel-loading buffer was added to each reaction and loaded onto a 4% non-denaturing gel and electrophoresis until the dye was three-fourths of the way down the gel. The gel was dried at 80 °C for 1 h and exposed to film overnight at −70 °C. The relative density of the protein bands was scanned by densitometry using Mylmage, and quantified by Labworks 4.0 software (UVP Inc.).

2.6. Transfection and assay of luciferase activity

SW620 human colon cells (2.5×10^5 cells/cm^2) were plated in 24-well plates and transiently transfected with pNF-κB-Luc plasmid (5x NF-κB; Stratagene, CA, USA) using a mixture of plasmid and lipofectAMINE PLUS in OPTI-MEN according to manufacture's specification

(Invitrogen, Carlsbad, CA, USA). The transfected cells were treated with TNF-α (150 U/ml) and different concentrations (0, 10, 20, 40 and 80 μM) of HCA for 8 h. Luciferase activity was measured by using the luciferase assay kit (Promega) according to the manufacturer's instructions (WinGlow, Bad Wildbad, Germany).

2.7. Immunofluorescent labeling and scanning-laser confocal microscopy

SW620 cancer cells (2.5×10^5 cells/cm^2) were cultured on a chamber slide (Lab-Tak II chamber slider system, Nalge Nunc Int., Naperville, IL, USA), fixed in 4% paraformaldehyde, membrane-permeabilized by exposure for 30 min to 0.1% Triton X-100 in phosphate-buffered saline, and placed in blocking serum (5% bovine serum albumin in phosphate-buffered saline) at room temperature. Cells were then exposed to primary goat polyclonal antibody for p50 (1:100 dilution) overnight at 4 °C, after three washes with ice-cold phosphate-buffered saline, followed by exposure with an anti-goat biotinylated secondary antibody (Molecular Probes, Eugene, USA) for 1 h at room temperature. Immunofluorescence images were acquired using a confocal laser-scanning microscope (dual wavelength scan, MRC1024, Bio-Rad, Hercules, CA, USA) with a 630× magnification.

2.8. Detection of apoptosis

SW620 cancer cells (2.5×10^5 cells/cm^2) were cultured on a chamber slide (Lab-Tak II chamber slider system, Nalge Nunc Int., Naperville, IL, USA), fixed in 4% paraformaldehyde, membrane-permeabilized by exposure for 30 min to 0.1% Triton X-100 in phosphate-buffered saline at room temperature. Cells were then exposed to DAPI solution. Apoptotic cells were determined by the morphological changes after 4,6-diamino-2-phenylindole (DAPI) staining under fluorescence microscopic observation (DAS

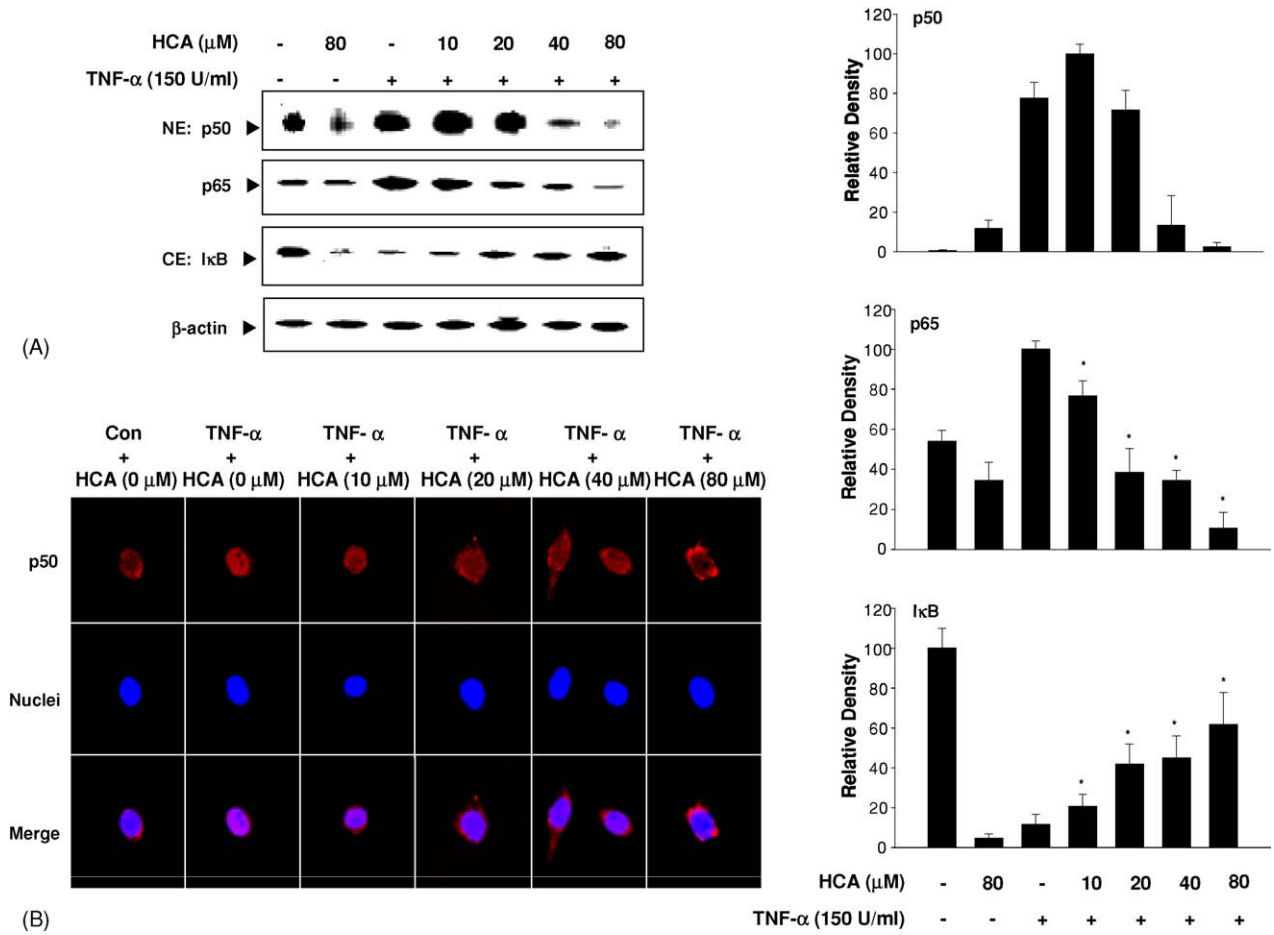

Fig. 2. Effect of HCA on p50, p65, and IκB-α levels as assessed by Western blot. (A) The cells were treated with 150 U/ml of TNF-α only or TNF-α plus different concentrations (0, 10, 20, 40 and 80 μM) of HCA at 37 °C for 1 h. Nuclear or cytosolic proteins (50μg) extracted after treatment were subjected to 12% SDS–PAGE. The expression of p50, p65, IκB-α and β-actin proteins were detected by Western blotting using specific antibodies, β-actin protein here was used as an internal control. Quantification of band intensities from three independent experimental results was determined by a densitometry (Imaging System). The density ratios of proteins expression were calculated, and the mean value in the untreated control group was set to 100. All values represent means ± S.E. of three independent experiments performed in triplicate. $^*P < 0.05$ indicates statistically significant differences from the TNF-α-treated group. (B) SW620 cancer cells were treated with 10–80 μM HCA in the presence of TNF-α at 37 °C for 1 h. Cells were washed, fixed, and the intracellular location of p50 was determined by immunofluorescence using an anti-p50 antibody. Pictures were taken with a confocal scanning microscope (magnification, 630×).

microscope, 200×: Leica Microsystems Inc., Deefield, IL, USA). For each determination, three separate 100-cell counts were scored. Apoptosis was expressed as a percentage calculated from the number of cells with apoptotic nuclear morphology divided by the total number of cells counted.

2.9. Data analysis

Statistical analysis data were analyzed using one-way analysis of variance followed by Tukey test as a post hoc test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. HCA inhibited TNF-α-induced NF-κB activation

There is evidence that HCA has an α,β-unsaturated carbonyl structure (Fig. 1A), which has been demonstrated to bind NF-κB, thereby inhibit its activity. We were interested in whether this substance inhibits colon cancer cell growth by inactivation of NF-κB because activation of NF-κB is a critical in cancer cell survival. We first examined the effect of HCA on TNF-α-induced NF-κB activation. SW620 colon cells were incubated with TNF-α and different concentrations of HCA. The nuclear proteins were extracted and the DNA-binding activity of NF-κB was determined by EMSA. Maximum activation of NF-κB was seen in 2 h after treatment with TNF-α (data not shown). In dose–response study, we found that the highest activity of NF-κB was seen after treatment of 150 U/ml TNF-α (data not shown). The DNA-binding activity of NF-κB was next determined in the cells treated with the combination of TNF-α (150 U/ml) and HCA. As shown in Fig. 1B, HCA (80 μM) alone did not activate NF-κB, but it inhibited TNF-α-induced NF-κB activation in a dose-dependent manner (0–80 μM). This DNA activity was confirmed by competition assay as well as by super shift assay (data not shown).

To further investigate the action mechanisms of HCA on the inhibition of TNF-α-induced NF-κB activation, we analyzed the effect of HCA on p50 and p65 nuclear translocation, and IκBα protein degradation. As shown in Fig. 2A, HCA treatment dose-dependently prevented the p50 and p65 nuclear translocation induced by TNF-α. HCA also inhibited the TNF-α-induced IκBα protein degradation in a dose-dependent manner. The increase of IκB expression could be related with increase of IκB release since HCA did not increase its expression (data not shown). The inhibitory effect of HCA on the translocation of p50 was also confirmed by confocal microscope analysis (Fig. 2B). In the unstimulated state, p50 was localized exclusively to the cytoplasm. However, much higher level of p50 was found in the nucleus after stimulation with TNF-α. HCA resulted in restore of cytoplasmic staining of p50 in dose-dependent manner. These results indicate that HCA prevents nuclear translocation of p50, thereby inhibits DNA-binding activity of NF-κB.

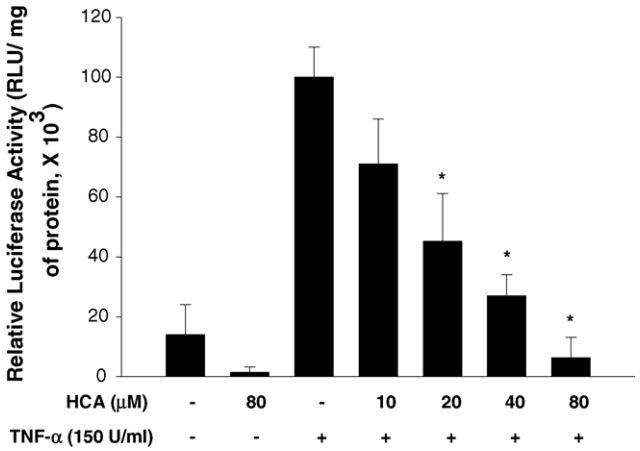

Fig. 3. Effect of HCA on TNF-α-induced NF-κB-dependent luciferase activity in SW620 cells. SW620 cells were transfected with pNF-κB-Luc plasmid (5x NF-κB) and then activated with TNF-α (150 U/ml) alone or TNF-α plus different concentrations (10, 20, 40 and 80 μM) of HCA at 37 °C, and then the luciferase activity was determined. All values represent means ± S.D. of three independent experiments performed in triplicate. RLU is relative to luciferase activity transfected unstimulated cells. $^{*}P < 0.05$ indicates statistically significant differences from the TNF-α-treated group.

3.2. HCA inhibited TNF-α-induced NF-κB transcriptional activation

To determine the effect of HCA on TNF-α-induced NF-κB-dependent reporter gene expression, we transiently transfected the cells with NF-κB-regulated luciferase reporter construct, and then the transfected cells were stimulated TNF-α alone or the combination of TNF-α and HCA. Consistent with the inhibitory effect on NF-κB DNA-binding activity, HCA (10–80 μM) inhibited TNF-α-induced NF-κB luciferase activity dose-dependently (Fig. 3).

3.3. HCA inhibited in TNF-α-induced SW620 human colon cancer cell growth

When chronically produced, TNF-α is thought to act as a tumor promoter that contributes to the tissue remodeling which is necessary for tumor growth and metastasis [29]. To investigate the inhibitory effect of HCA on the TNF-α-induced SW620 human colon cancer cell growth, we analyzed cell growth by MTT assay and by direct cell counting. Morphological observation showed that the cells were gradually reduced cell size, and changed to round single cell shape in a dose manner by the treatment of HCA (Fig. 4A). HCA also inhibited cell growth and cell viability in dose (0–80 μM) and time (0–48 h) dependent manners in TNF-α-induced SW620 human colon cancer cells (Fig. 4B). In order to determine whether apoptotic cell

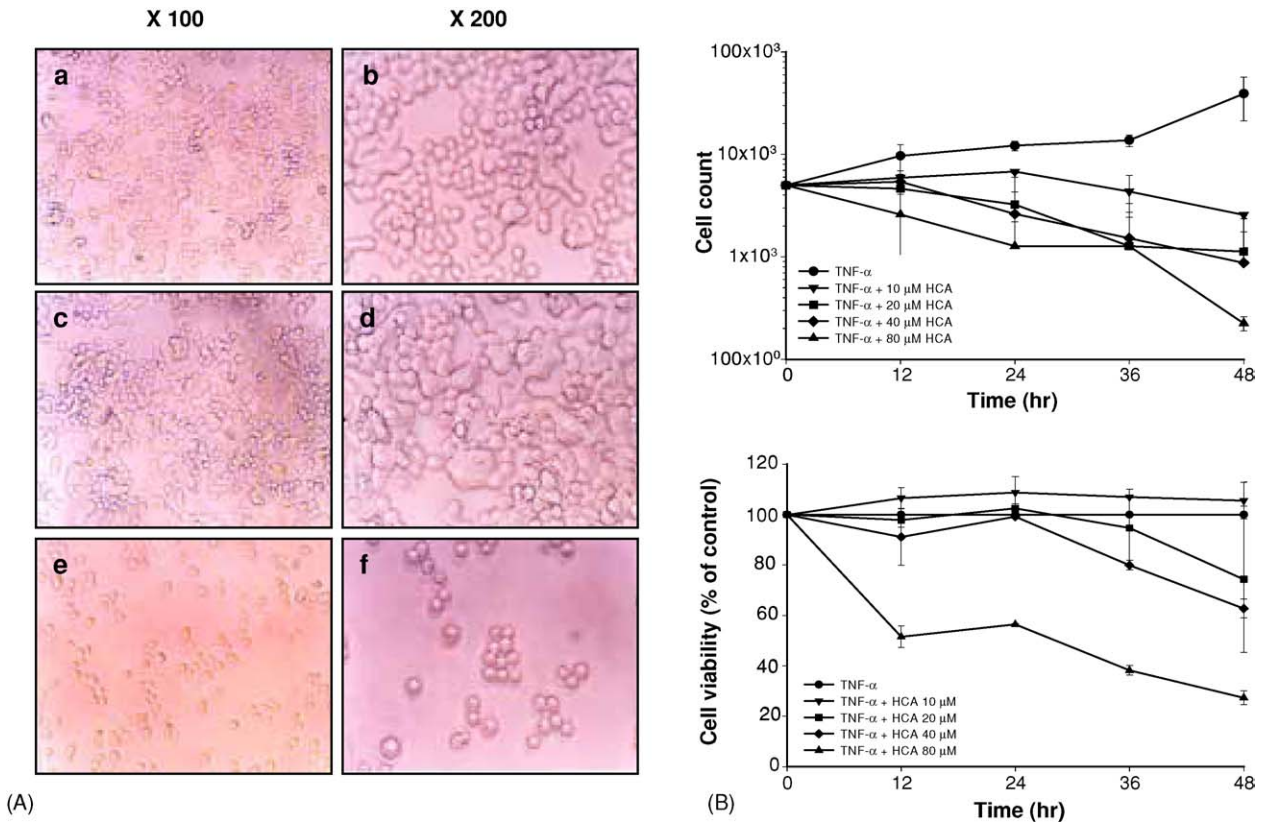

Fig. 4. Morphological changes and cell viability of SW620 cells by HCA. (A) Morphorogical changes were observed under microscope (magnification, 100× and 200×). a and b, non-treated group; c and d, TNF-α-treated control group; e and f, TNF-α + 40 μM HCA. (B) Cell viability was determined by MTT assay or by directly counting as described in Section 2. Values are mean ± S.D. of three experiments, with triplicate of each experiment.

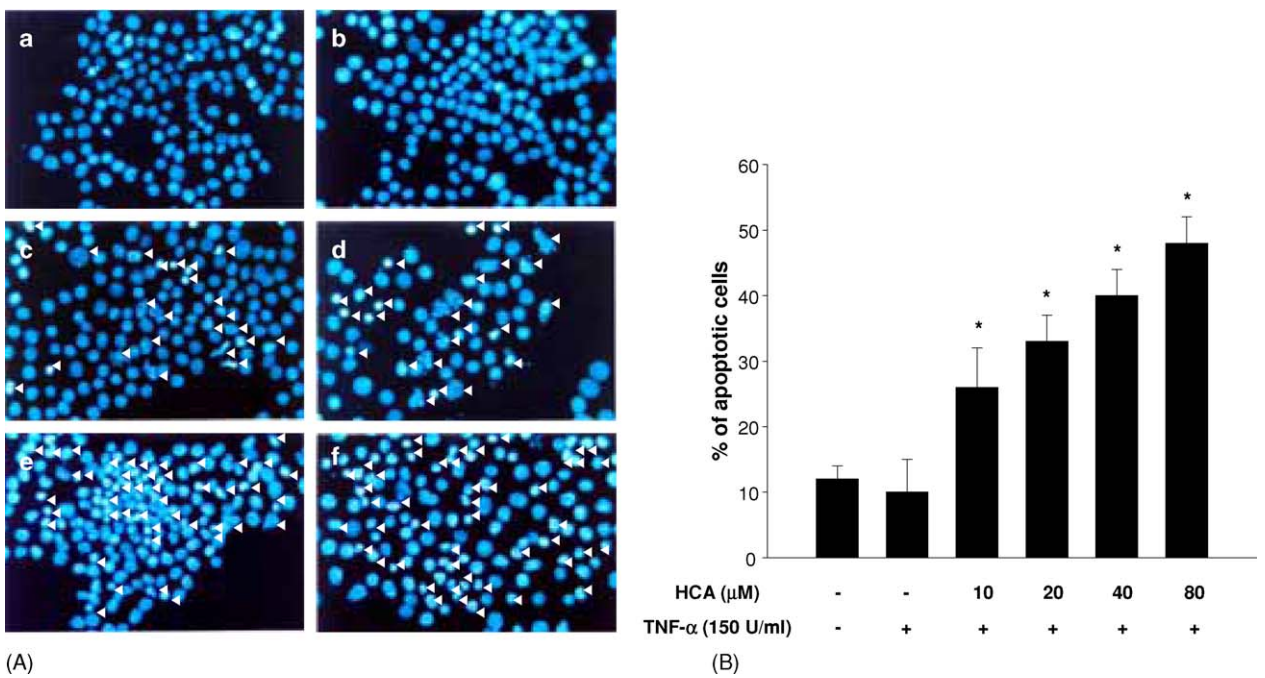

Fig. 5. Effects of HCA on the induction of apoptosis of TNF-α-induced SW620 cells analyzed by DAPI staining. The apoptotic cells were examined by fluorescence microscopy. Treatment of HCA for 24 h caused apoptosis characterized by marked chromatin condensations, small membrane-bound bodies (apoptotic bodies), cytoplasmic condensations, and cellular shrinkage. The cell indicated by arrows is an example of apoptotic cells (magnification, 200×). Apoptotic cells were estimated by direct counting of fragmented nuclei after DAPI staining. The value are means ± S.D. of three experiments, with triplicate of each experiment. $^{*}P < 0.05$ indicates statistically significant differences from the TNF-α-treated group. a, non-treated group; b, TNF-α-treated control group; c, TNF-α + 10 μM HCA; d, TNF-α + 20 μM HCA; e, TNF-α + 40 μM HCA; and f, TNF-α + 80 μM HCA.

death contributed to the cell growth inhibition, we evaluated changes in the chromatin morphology of TNF-α-induced SW620 cells using DAPI staining (Fig. 5A). Apoptosis determined after 24 h treatment was increased in a dose-dependent manner in SW620 human colon cancer cells (Fig. 5B).

3.4. HCA induced the expression of apoptotic regulatory proteins

NF-κB can regulate genes controlling apoptotic cell death. NF-κB activation in cancer cells correlates with resistance to apoptosis and increased levels of anti-apoptotic proteins. Anticancer drugs are considered to mediate cell death by activating key elements of the apoptosis program relating NF-κB. To figure out the relationship between the induction of apoptosis by HCA and expression of apoptotic gene expression, expression of apoptosis related proteins was investigated. Expression of pro-apoptotic proteins, Bax, and active form of caspases 3 was increased but the expression of anti-apoptotic protein bcl-2 was decreased. Moreover, the ratio of Bax/$Bcl_2$was significantly increased after HCA treatment in a dose-dependent manner (Fig. 6).

3.5. Involvement of ERK pathway in HCA-induced cell growth and NF-κB inhibition

Accumulating evidence indicates that NF-κB activation is modulated by MAPK family such as ERK, JNKs [16] as well as p38 MAPK [17]. The effects of HCA on the activation of mitogen-activated protein kinase were examined as a possible signal pathway. The expression of total ERK and JNK was decreased in the TNF-α-induced SW620 cells. However, only phosphorylated ERK expression was increased by HCA dose dependency (Fig. 7). These results show that among MAPKs, activation of ERK was involved with HCA-induced induction of apoptosis as well as inhibition of cell growth. We next investigated whether MEK1 inhibitor reversed HCA-induced cell

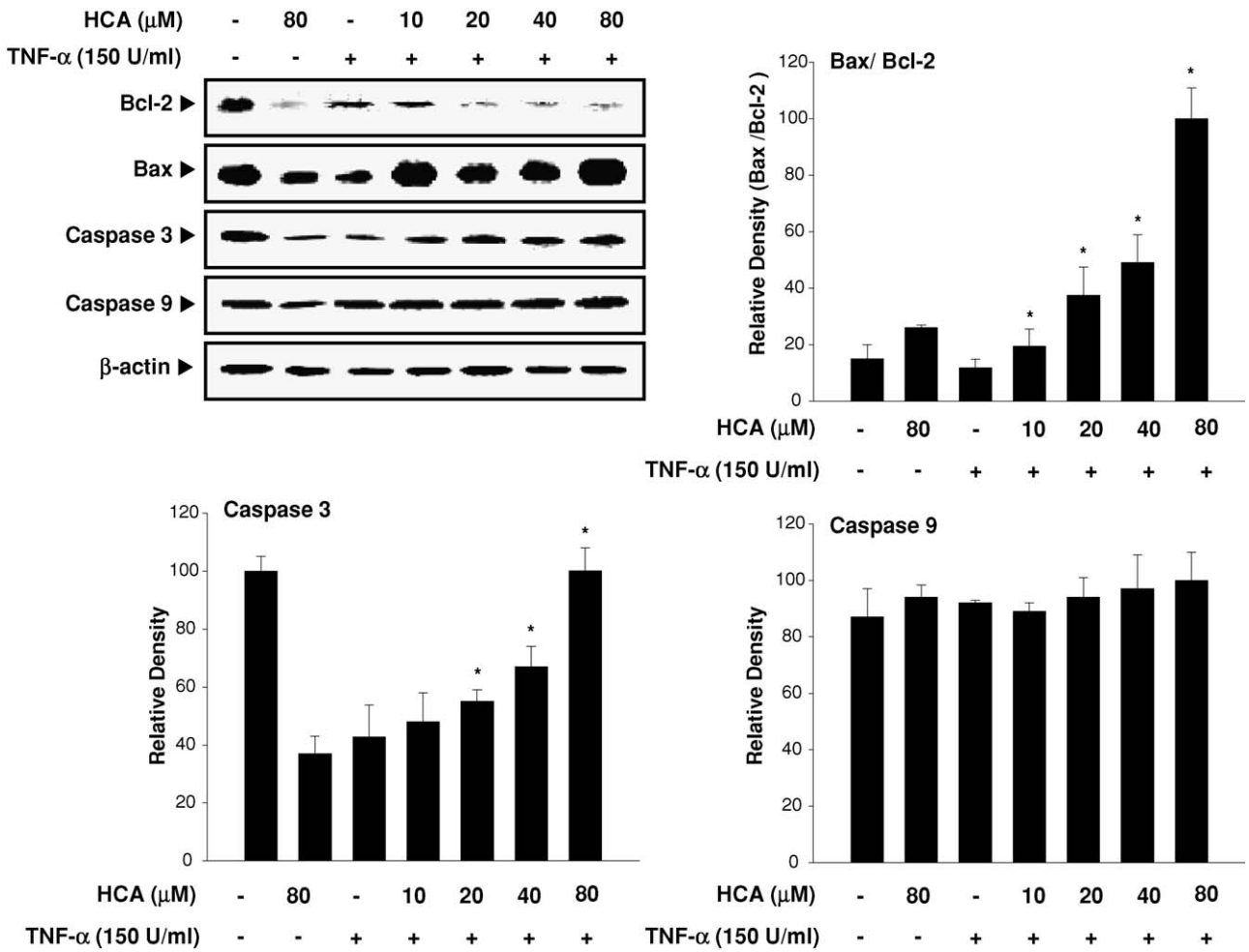

Fig. 6. Effect of HCA on TNF-α-induced expression of apoptosis regulatory proteins. The cells were treated with 150 U/ml of TNF-α only orTNF-α plus different concentrations (0, 10, 20, 40 and 80 μM) of HCA at 37 °C for 24 h. Equal amounts of total proteins (50 μg/lane) were subjected to 12% SDS–PAGE. Expression of Bcl-2, Bax, Caspase 3, Caspase 9 and β-actin were detected by Western blotting using specific antibodies, β-actin protein here was used as an internal control. Quantification of band intensities from three independent experimental results was determined by a densitometry (Imaging System). $^{*}P < 0.05$ indicates statistically significant differences from the TNF-α-treated group.

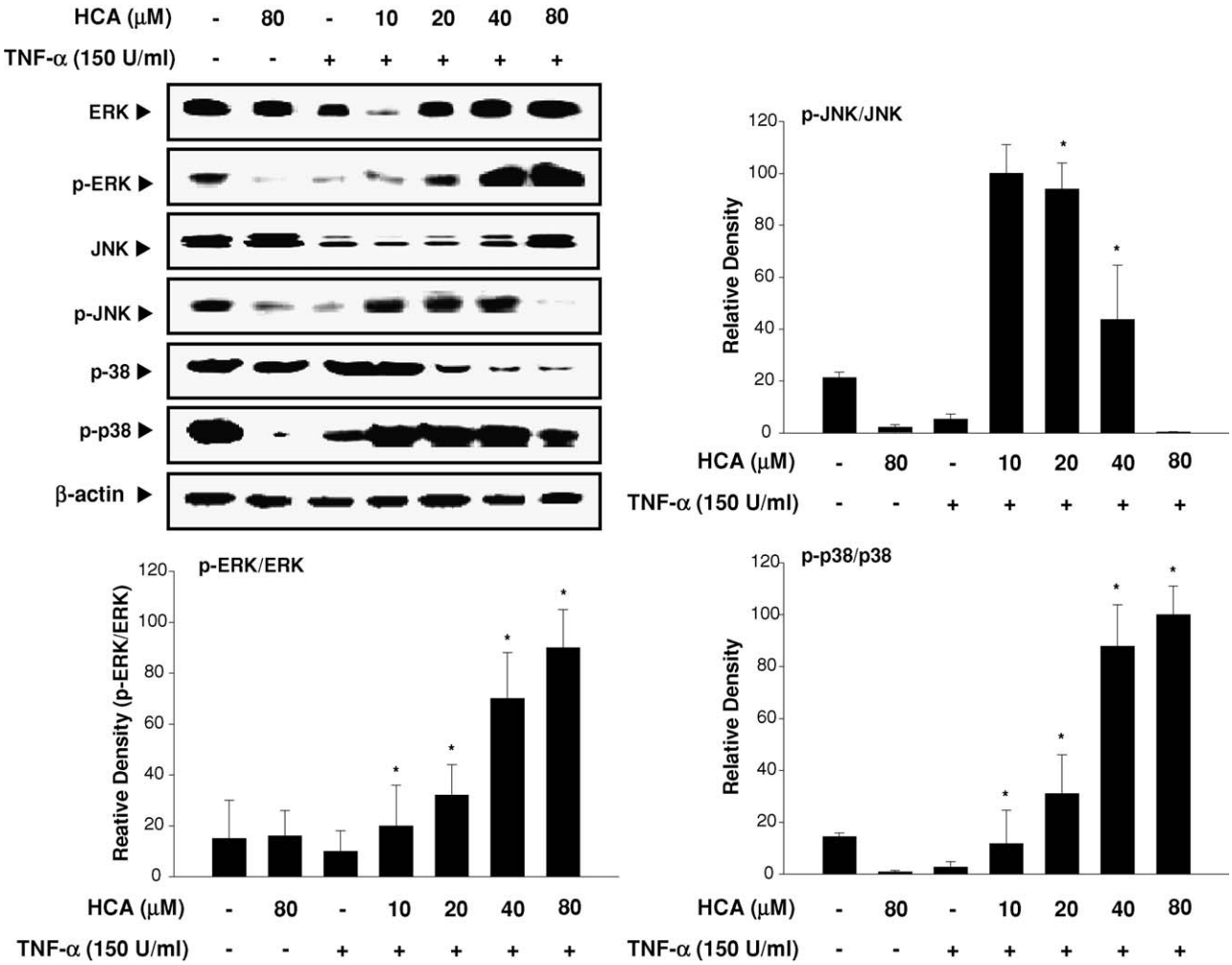

Fig. 7. Effect of HCA on TNF-α-induced ERK activity. The cells were treated with 150 U/ml of TNF-α only or TNF-α plus different concentrations (0, 10, 20, 40 and 80 μM) of HCA at 37 °C for 24 h. Equal amounts of total proteins (50 μg/lane) were subjected to 10% SDS–PAGE. The expressions of p-ERK, p-p38, p-JNK, ERK, p38, and JNK protein were detected by Western blotting using specific antibodies. Quantification of band intensities from three independent experimental results was determined by a densitometry (Imaging System). Data was described as means ± S.D. from three experiments performed in triplicate for p-ERK/ERK, p-p38/p38 and p-JNK/JNK. $^{*}P < 0.05$ indicates statistically significant differences from the TNF-α-treated group.

growth inhibition to further study the relevance of ERK pathway in HCA-inhibited cell growth. MEK1 inhibitor (ERK upstream kinase inhibitor), PD98059 partially reversed HCA-induced cell growth inhibition (Fig. 8A) but other MAP kinase inhibitors did not work (data not shown). PD98059 also reversed HCA-induced inhibition of NF-κB transcriptional activity (Fig. 8B).

4. Discussion

HCA has been shown to exert a potent anti-tumor effect in a number of different cell types, including colon cells [18,21]. In the present study, we demonstrated that HCA treatment resulted in inhibition of NF-κB activation in TNF-α-induced SW620 human colon cancer cells. This effect was accompanied by cell growth inhibition and induction of apoptosis. Recent evidence indicates that NF-κB signaling pathways are involved in the tumor development [30,31]. Constitutively activated NF-κB transcription factor have been associated with several aspects of tumorigenesis, including cancer cell proliferation, prevention of apoptosis, and increase of angiogenesis and metastasis potential [1]. NF-κB transcription factor is constitutively activated in human colorectal carcinoma tissue and human pancreatic cancer cells [32,33]. Therefore, activation of NF-κB may give favor circumstance for colon cancer cell growth. From this knowledge, transcription factor NF-κB can be specifically targeted to prevent colon cancer cell growth. The present data indicated that the inhibition of NF-κB by HCA might be at least a critical mechanism in the inhibition of SW620 human colon cancer cell growth. Inhibitory effect of transcriptional activity by HCA further supported this notion.

The mechanisms how HCA can interfere NF-κB activation is not clear. However, it is known that HCA is a α,β-unsaturated carbonyl compounds, which can react with nucleophiles, especially with cysteine sulfhydryl groups of

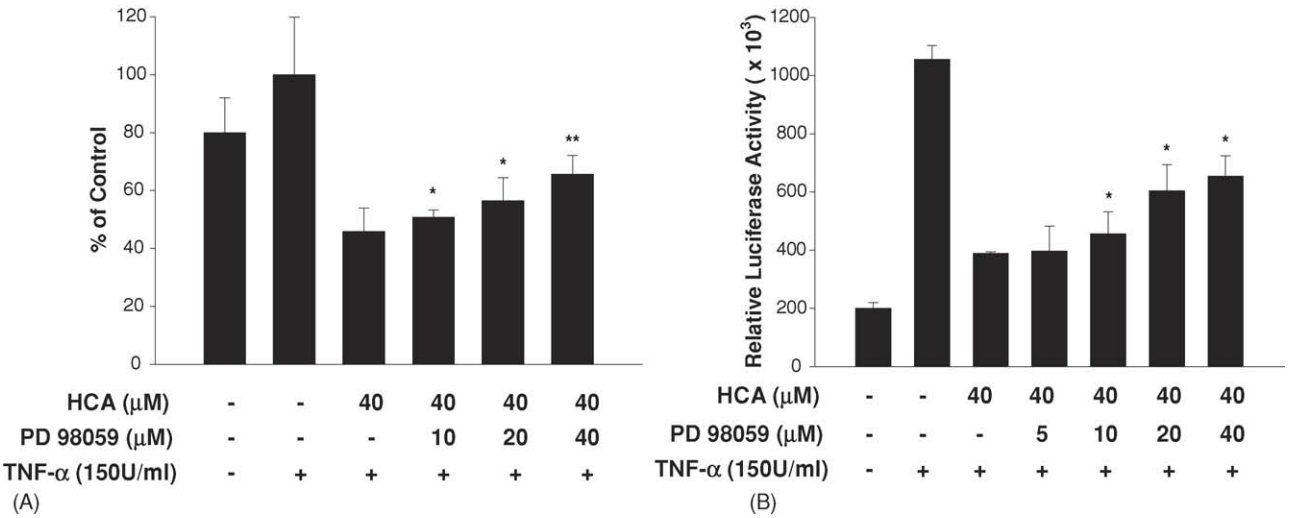

Fig. 8. Effect of ERK inhibitor (PD98059) in cell growth and NF-κB transcriptional activation. (A) TNF-α-induced SW620 human colon cancer cells were treated with HCA (40 μM) for 24 h and assayed for cell viability by MTT assay. Where indicated, cells were pretreated with PD98059 (10, 20 and 40 μM) for 1 h before the addition of HCA. Data was described as means ± S.D. from three experiments performed in triplicate for cell growth. $^{*}P < 0.05$ indicates statistically significant differences from the TNFα + HCA-treated group. (B) SW620 cells were transfected with pNF-κB-Luc plasmid (5x NF-κB) and then activated with TNF-α (150 U/ml). SW620 human colon cancer cells were treated with HCA (40 μM) for 24 h and then the luciferase activity was determined. Where indicated, cells were pretreated with PD98059 (5, 10, 20 and 40 μM) for 1 h before the addition of HCA. All values represent means ± S.D. of three independent experiments performed in triplicate. RLU is relative to luciferase activity transfected unstimulated cells. $^{*}P < 0.05$ indicates statistically significant differences from the TNFα + HCA-treated group.

target molecules, in Michael-type addition. Several studies have shown that an α,β-unsaturated carbonyl compounds react directly with cysteine sulfhydryl groups of NF-κB subunit or alters expression of the NF-κB target gene product [37,38]. In NF-κB/p65, cysteine L1 (L1-Cys, position 38) participates in DNA-binding by forming a hydrogen bond with the sugar/phosphate backbone of the κB-DNA motif. Therefore, it is highly possible that HCA resulted in decrease of DNA-binding activation of NF-κB through reacting directly with cysteine sulfhydryl groups of NF-κB subunit molecules. Data presented herein that HCA treatment significantly inhibits NF-κB activation by reducing the degradation of lκB. These results suggest another possibility that HCA inhibited the upstream proteins of lκB such as IKKs or 26s proteasome. Interestingly, three other IKKβ inhibitors have been recently shown to target the cysteine residue of IKKβ [34,35]. Similar mechanism was reported. For example; the α,β unsaturated carbonyl group of prostaglandin A_1 was shown to be essential for the covalent modification of IKKβ, putatively via interaction with cysteine 179 [35]. Similarly, modification of cysteine 179 of IKKβ has been proposed to mediate the pathological effects of arsenite and parthenolide [36]. Thus, the α,β unsaturated carbonyl group of HCA may be essential for the covalent modification of NF-κB or/and IKKβ via interaction with cysteine as above compounds. Alternatively, the aldehyde of HCA has a possibility that react with the catalytic hydroxyl or thiol groups in the active sites of those enzymes to form a reversible hemi (thio) acetal as peptide aldehyde [39].

Protein comprising the mitogen-activated protein kinase family constitutes important mediators of signal transduction processes that serve to coordinate the cellular response to a variety of extra stimuli. MAPKs regulate NF-κB activity by multiple mechanisms. ERK induces site-specific phosphorylation of IκB-α in HeLa cells and directly activates the IKK complex [16]. Many agents that activate MAPKs also activate NF-κB, suggesting that cross talk occurs between these pathways [40]. The ERK pathways play a major role in regulating cell growth and differentiation, being highly induced in response to growth factors, cytokines, and phorbol esters [12]. ERK is activated in cisplatin-induced apoptosis of HeLa cells [13]. ERK activation was also found in gemcitabine-induced Bcl-2 pathway-dependent apoptotic cell death in human NSCLC H1299 cells [14]. Similar to these findings and consistent with cell growth inhibition, treatment of HCA to SW620 cells activated ERK but not other MAP kinases. Moreover, inhibitor of ERK partially blocked HCA-induced cell growth inhibition and NF-κB transcriptional activity. These results show that ERK-dependent NF-κB inactivation were involved in HCA-induced cell growth inhibition.

HCA treatment for 24 h in TNF-α-induced SW620 human colon cancer cells resulted in a dose-dependent increase of apoptosis. It was also found that consistent with the increase of apoptosis, the expression of apoptotic proteins active caspase 3, Bax was dose dependency increased, but the anti-apoptotic protein Bcl-2 was decreased. Apoptosis is an important mechanism to eliminate unwanted cells in a wide variety of physical processes, and deregulation of this process is implicated in pathogenesis of many chronic diseases, including cancer and autoimmunity [41]. Proteins in this process, including Bcl-2 and Bcl-X_L, inhibit apoptosis, while others such as

Bax and Bak promote apoptosis [42]. Hence, an alteration in the levels of anti- and pro-apoptotic proteins is likely to influence apoptosis. NF-κB activation in cancer cells correlates with resistance to apoptosis and increased levels of anti-apoptotic Bcl-2 family proteins [43]. In this study, treatment of HCA in cells inhibited NF-κB activation and IκB degradation, and reduced Bcl-2 protein level. Caspases are cysteine proteases that play a critical role in the execution of apoptosis [44]. These data suggest that HCA-induced apoptosis through inhibited NF-κB activation could be related with alternation of the expression of cell death regulatory proteins.

In conclusion, the current study showed that HCA exerts its cell growth inhibition effect by inhibition of NF-κB in an ERK pathway-dependent manner in TNF-α-induced SW620 human colon cancer cells. Therefore, this data suggest that HCA can be a useful agent for prevention of cancer cell growth.

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