

Molecular mechanisms of curcumin-induced cytotoxicity: induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-X_L and IAP, the release of cytochrome *c* and inhibition of Akt

Ju-Hyung Woo¹, Young-Ho Kim¹, Yun-Jung Choi¹,
Dae-Gon Kim², Kyung-Seop Lee², Jae Hoon Bae³, Do Sik
Min⁴, Jong-Soo Chang⁵, Yong-Jin Jeong⁶, Young Han
Lee⁷, Jong-Wook Park¹ and Taeg Kyu Kwon^{1,8}

¹Department of Immunology, School of Medicine, Keimyung University, 194 DongSan-Dong Jung-Gu, Taegu, 700-712, ²Department of Urology, College of Medicine, Dongguk University, Kyungju, ³Department of Physiology School of Medicine, Keimyung University, Taegu, ⁴Department of Physiology, College of Medicine, The Catholic University of Korea, Seoul, 137-701, ⁵Department of Life Science, Daejin University, Pochon-gun, Kyeonggido, ⁶Department of Food Science and Technology, Keimyung University and ⁷Department of Biochemistry, College of Medicine, Yeungnam University, Taegu, South Korea

⁸To whom correspondence should be addressed
Email: kwontk@dsmc.or.kr

Curcumin, a natural, biologically active compound extracted from rhizomes of *Curcuma* species, has been shown to possess potent anti-inflammatory, anti-tumor and anti-oxidative properties. The mechanism by which curcumin initiates apoptosis remains poorly understood. In the present report we investigated the effect of curcumin on the activation of the apoptotic pathway in human renal Caki cells. Treatment of Caki cells with 50 μ M curcumin resulted in the activation of caspase 3, cleavage of phospholipase C- γ 1 and DNA fragmentation. Curcumin-induced apoptosis is mediated through the activation of caspase, which is specifically inhibited by the caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone. Curcumin causes dose-dependent apoptosis and DNA fragmentation of Caki cells, which is preceded by the sequential dephosphorylation of Akt, down-regulation of the anti-apoptotic Bcl-2, Bcl-X_L and IAP proteins, release of cytochrome *c* and activation of caspase 3. Cyclosporin A, as well as caspase inhibitor, specifically inhibit curcumin-induced apoptosis in Caki cells. Pre-treatment with *N*-acetyl-cysteine, markedly prevented dephosphorylation of Akt, and cytochrome *c* release, and cell death, suggesting a role for reactive oxygen species in this process. The data indicate that curcumin can cause cell damage by inactivating the Akt-related cell survival pathway and release of cytochrome *c*, providing a new mechanism for curcumin-induced cytotoxicity.

Introduction

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major constituent of turmeric powder extracted from the rhizomes of the plant *Curcuma longa* found

Abbreviations: DEVD-pNA, Asp-Glu-Val-Asp-chromophore *p*-nitroanilide; H₂DCFDA, 2,7-dichlorodihydrofluorescein diacetate; NAC, *N*-acetyl-cysteine; PLC- γ 1, phospholipase C- γ 1; ROS, reactive oxygen species; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

in south and southeast tropical Asia. It has been used for centuries in indigenous medicine for the treatment of a variety of inflammatory conditions and other diseases (1). Several studies in recent years have shown that curcumin is a potent inhibitor of tumor initiation *in vivo* (2,3) and possesses anti-proliferative activities against tumor cells *in vitro* (4). Curcumin is also a potent chemopreventive agent inhibiting tumor promotion against skin, oral, intestinal and colon carcinogenesis (5,6).

The cancer chemopreventive activity of curcumin in human has yet to be confirmed, however. Recent evidence indicates that curcumin suppresses a number of key elements in cellular signal transduction pathways. Prominent among the signaling events inhibited by curcumin are phosphorylations catalyzed by protein kinases (7), c-Jun/AP-1 activation (8) and prostaglandin biosynthesis (9). All of these inhibitory actions of curcumin require concentrations of the agent in the 10–100 μ M range. Recently, it has been reported that curcumin treatment resulted in the production of reactive oxygen species (ROS) (10,11). ROS is involved in the initiation and progression of a variety of human diseases (12), including renal ischemia/reperfusion injury and in toxicities associated with chemical exposure. The kidney or kidney-derived cells have an increased susceptibility towards agents generating oxygen reactive species in terms of apoptogenicity and proliferation, and ROS play an important role in the pathogenesis of a variety of renal diseases (13). Moreover, renal cell carcinoma remains one of the most drug-resistant malignancies in humans and is a frequent cause of cancer mortality (14). Thus, we investigated the relation between the curcumin-induced oxidative response and toxicity in renal carcinoma cells, human renal Caki cells.

Curcumin was also shown to inhibit the *in vitro* growth of a number of human cancer cell lines (15), the cellular and molecular mechanisms underlying curcumin-induced apoptosis are not yet well defined.

To explore the mechanism of the chemopreventive effects of curcumin, we have tested whether curcumin can induce apoptosis in tumor cells and examined the involvement of these pro- and anti-apoptotic members of the Bcl-2 and IAP family in materializing such apoptogenic effect. We have further analyzed that curcumin activates mitochondria-mediated apoptotic pathway in tumor cells. Curcumin induces cytochrome *c* release, which activates pro-caspase 3 and DNA fragmentation. Moreover, antioxidant *N*-acetyl-cysteine (NAC) inhibits curcumin-induced apoptosis and prevents the release of cytochrome *c* from the mitochondria. The data presented here demonstrate that the cytotoxicity of curcumin is due to the induction of apoptosis that is mediated by the direct release of cytochrome *c* and the subsequent activation of caspases.

Materials and methods

Cells and materials

Human renal carcinoma Caki cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium,

containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100 µg/ml gentamicin. Curcumin was directly added to cell cultures at the indicated concentrations. Anti-cIAP1, anti-cIAP2, anti-Bcl-2, anti-Bcl-X_L and anti-Bax, anti-actin and anti-HSP70 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the following proteins were purchased from the indicated suppliers: PARP from Boehringer Mannheim (Indianapolis, IN), cytochrome *c* from PharMingen (San Diego, CA) and XIAP from R&D systems (Minneapolis, MN). Asp-Glu-Val-Asp-chromophore *p*-nitroanilide (DEVD-pNA), benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) and curcumin were purchased from Biomol (Plymouth Meeting, PA).

Western blotting

Cellular lysates were prepared by suspending 1×10^6 cells in 100 µl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl₂, 0.1% Triton X-100, 25 mM MOPS, 100 µM phenylmethylsulfonyl fluoride and 20 µM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA). Detection of specific proteins was carried out with an enhanced chemiluminescence western blotting kit according to the manufacturer's instructions.

DNA fragmentation assay

After treatment with curcumin, Caki cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10 000 *g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol–chloroform–isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 µg/ml of ethidium bromide.

Measurement of reactive oxygen species

The intracellular accumulation of ROS was determined using the fluorescent probes (2,7-dichlorodihydrofluorescein diacetate) H₂DCFDA. H₂DCFDA was commonly used to measure H₂O₂ (16), but it is now accepted that this probe is also sensitive to other peroxides (17). With this aim, 1 h prior to treatment with the cytotoxic agents the cells were collected by centrifugation, resuspended in DMEM medium without red phenol, and loaded with either 5 µM H₂DCFDA. The fluorescence was measured at the desired time intervals by flow cytometry. Control cells were subjected to the same manipulation, except for treatment with the curcumin.

Caspase 3 activity assay

To evaluate caspase 3 activity, cell lysates were prepared after their respective treatment with curcumin. Assays were performed in 96 well microtitre plates by incubating 20 µg of cell lysates in 100 µl of reaction buffer (1% NP-40, 20 µM Tris–HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing the caspase 3 substrate (DEVD-pNA) at 5 µM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Analysis of cytochrome *c* release

Cells (2×10^6) were harvested, washed once with ice-cold phosphate-buffered saline and gently lysed for 2 min in 80 µl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris–HCl, pH 7.2, 1 mM dithiothreitol, 10 mM KCl, 1.5 mM MgCl₂, 5 µg/ml pepstatin A, 10 µg/ml leupeptin, 2 µg/ml aprotinin). Lysates were centrifuged at 12 000 *g* at 4°C for 10 min to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). The resulting cytosolic fractions were used as the cytosolic fraction for detecting cytochrome *c* and the resulting pellets were used as a positive control for mitochondrial protein cytochrome *c* oxidase subunit IV.

RNA isolation and reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was isolated according to a published method (18). Single-strand cDNA was synthesized from 2 µg of total RNA using M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD). The cDNA for Akt and actin were amplified by PCR with specific primers. The sequences of the sense and antisense primers for Akt were 5'-CAACTTCTCTGTGGCGCAGTG-3' and 5'-GACAGGTGGAAGAAGACTCG-3', respectively. Conditions for PCR reaction were 1× (94°C, 3 min); 30× (94°C, 45 s; 58°C, 45 s and 72°C, 1 min); and 1× (72°C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Statistical significance

All experiments were repeated at least three times with different cells. Values are the mean ± SD of these experiments. Significance between experimental values was determined by two-way analysis of variance.

Results

Cytotoxic effect of curcumin on Caki cell death

To verify curcumin-induced cell toxicity, we first examined the changes in cell morphology after curcumin exposure. As shown in Figure 1A, exposure to 50 µM curcumin for 24 h causes Caki cells to develop characteristic features of cell shrinking, rounding and partial detachment, thus demonstrating the lobulated appearance of apoptotic cells (Figure 1A). We also examined the effects of different concentrations of curcumin on cell viability on Caki cells. After a 24 h treatment, survival was inversely correlated with curcumin concentration. At the concentration we used (50 and 75 µM), significant loss of viability was detectable during the 36 h of treatment. After treatment with curcumin 50 µM for 24 and 36 h, 55 and 18% of the cells survived in culture, respectively (Figure 1B). These results indicate that curcumin-mediated reduction of cell viability was dose- and time-dependent.

To confirm the induction of apoptosis, Caki cells were treated with various concentrations of curcumin, and DNA was isolated and analyzed by agarose gel electrophoresis. These experiments demonstrated that DNA from Caki cells treated with various concentrations of curcumin for 24 h, a typical ladder pattern of internucleosomal fragmentation was observed in high concentration of curcumin (Figure 1C).

Caspase mediate curcumin-induced apoptosis

Recent studies have identified caspases as important mediators of apoptosis induced by various apoptotic stimuli (19). To determine the roles of caspases in curcumin-induced apoptosis, we measured the activity of caspase 3 in curcumin-treated Caki cells by western blot analysis. Because caspase 3 is activated by proteolytic processing of the 32 kDa form into two smaller subunits, activity of caspase 3 can be detected by a decrease in pro-enzyme level using western blot analysis and a proteolytic activity with a chromogenic substrate. As shown in Figure 2A, treatment with curcumin results in decreased levels of pro-caspase 3 in Caki cells exposed to 50–100 µM curcumin for 24 h. Subsequent western blotting demonstrated proteolytic cleavage of phospholipase C-γ1 (PLC-γ1), a downstream target of activated caspase 3 *in vivo* (20), in Caki cells after 24 h of 50 µM curcumin. This cleavage of PLC-γ1 is dose-dependent in Caki cells (Figure 2A).

To further investigate and quantify the proteolytic activity of caspase 3, we performed an *in vitro* assay based on the proteolytic cleavage of DEVD-pNA by caspase 3 into the pNA. Caki cells demonstrate a 4-fold increase in DEVD-pNA cleavage after 24 h exposure to 100 µM curcumin (Figure 2B). We also tested the effect of curcumin on induction of apoptosis in leukemia U937 cells, and found that curcumin induces PLC-γ1 cleavage and activation of caspase 3 in U937 cells at even lower concentration than what is required for these effects in Caki cells (Figure 2C and D).

To address the significance of caspase activation in curcumin-induced apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk. Curcumin strongly stimulates caspase 3 protease activities, but 50 µM z-VAD-fmk pretreatment abolishes 75 µM curcumin-induced caspase 3 activities (Figure 3A). Furthermore, curcumin treatment of Caki cells generates a 60 kDa cleavage product of PLC-γ1 and cleavage of Bax protein. However, z-VAD-fmk pre-treated cells significantly inhibit cleavage of PLC-γ1 and Bax (Figure 3B).

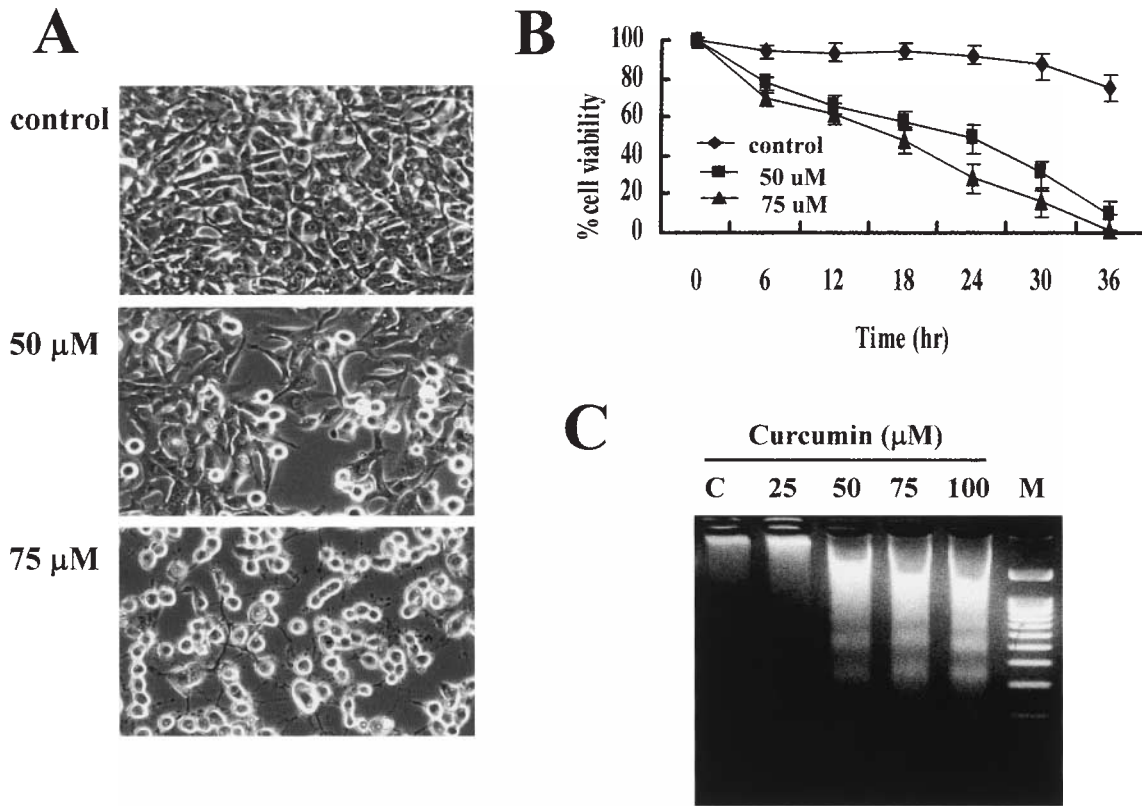


Fig. 1. Effect of curcumin on morphological characteristics, viability and DNA fragmentation of Caki cells. (A) Caki cells were treated with vehicle, 50 μM curcumin, and 75 μM curcumin for 24 h. Magnification, $\times 200$. (B) Caki cells were treated with different concentrations of curcumin for various time periods. Cell death was determined by using trypan blue exclusion. Data shown are means \pm SD ($n = 3$). (C) DNA fragmentations in Caki cells were treated for 24 h with indicated concentrations of curcumin. Fragmented DNA was extracted and analyzed on 2% agarose gel.

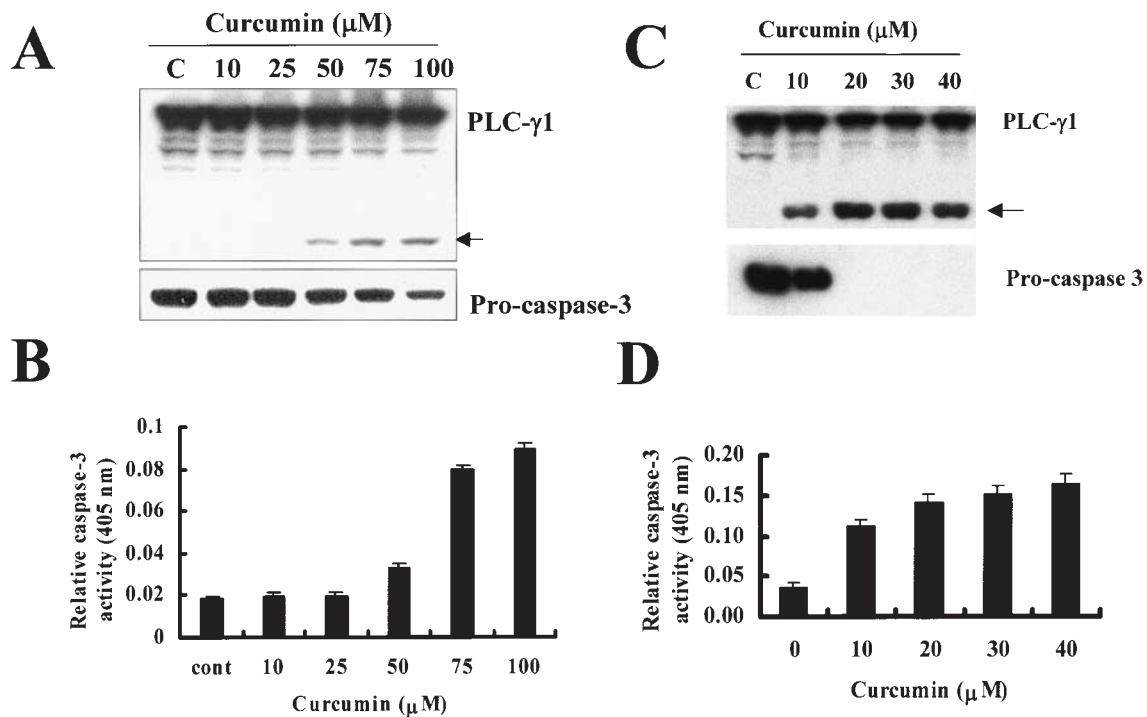


Fig. 2. Effect of curcumin on caspase-specific cleavage of PLC- $\gamma 1$, and caspase 3 activity in Caki and U937 cells. Caki cells (A) and U937 cells (C) were treated with the indicated concentrations of curcumin. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blot for pro-caspase 3 and PLC- $\gamma 1$. The proteolytic cleavage of PLC- $\gamma 1$ is indicated by an arrow. Caki cells (B) and U937 cells (D) were treated with indicated concentrations of curcumin for 24 h and harvested in lysis buffer. Enzymatic activities of caspase 3 were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (DEVD-pN) in a 100 μl assay buffer for 2 h at 37°C. The release of chromophore pNA was monitored spectrophotometrically (405 nm). Data shown are means \pm SD ($n = 3$).

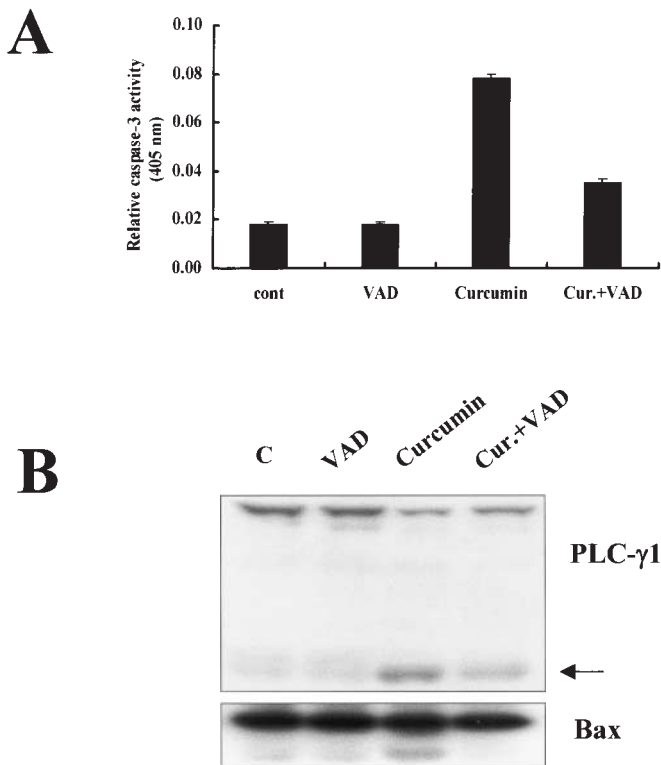


Fig. 3. Caspase-mediated apoptosis induced by curcumin. (A) Effects of z-VAD-fmk on curcumin-induced caspase 3 activation. Caki cells were incubated with 50 μ M z-VAD-fmk or solvent for 1 h before treatment with 75 μ M curcumin. Caspase activity was determined as described in Figure 2. Data shown are means \pm SD ($n = 3$). (B) Effect of z-VAD-fmk on cleavage of PLC- γ 1 and Bax. Caki cells were incubated with 50 μ M z-VAD-fmk or solvent for 1 h before treatment with 75 μ M curcumin. Equal amounts of cell lysates (40 μ g) were subjected to electrophoresis and analyzed by western blot for PLC- γ 1 and Bax. The proteolytic cleavage of PLC- γ 1 is indicated by an arrow.

The data clearly indicate that curcumin-induced apoptosis is associated with caspase activation.

Modulation of Bcl-2 and IAP protein families in curcumin-induced apoptosis in Caki cells

We also examined whether curcumin induces cell death by modulating the expression of Bcl-2 family members, which ultimately determine the cell's response to apoptotic stimuli. Treatment of Caki cells for 24 h with concentrations of curcumin that are sufficient to induce apoptosis does significantly alter the expression of the Bcl-2 and Bcl-X_L, but not Bax proteins (Figure 4A). These results indicate that the expression levels of Bcl-2 and Bcl-X_L proteins modulate apoptosis induced by curcumin in Caki cells.

To determine whether activity of caspase 3 is associated with the levels of caspase inhibitors in curcumin-induced apoptosis, we determined the expression levels of IAP family proteins in Caki cells after exposure to various concentrations of curcumin. As shown in Figure 4B, treatment with 50–100 μ M curcumin for 24 h results in decreased levels of cIAP1 and XIAP, but not cIAP2 in Caki cells. These results indicate that the elevated caspase 3 activity in curcumin treated Caki cells is correlated with down-regulation of cIAP1 and XIAP, but not cIAP2.

We then determined whether the expression levels of Bcl-2 and IAP family proteins are affected by the presence of a caspase-inhibitor. For these experiments, we pre-incubated Caki cells with the caspase inhibitor z-VAD-fmk. This pre-incubation of Caki cells with z-VAD-fmk resulted in a significant reduction of Bcl-2 and IAP family protein levels (Figure 4C).

Curcumin induces cytochrome c release in Caki cells

To examine the release of cytochrome *c* in curcumin-treated Caki cells, we conducted western blotting analysis with cytosolic fractions. These experiments demonstrated that treatment of curcumin in Caki cells significantly induces curcumin-mediated release of cytochrome *c* from the mitochondria into the cytoplasm (Figure 5A). This curcumin-induced increase in cytochrome *c* within the cytosol is dose-dependent.

To determine whether the observed cytochrome *c* release is indeed the trigger of cell death, or merely a consequence of caspase activation, we performed cytochrome *c* release analysis in caspase-inhibitor treated Caki cells. The release of cytochrome *c* from mitochondria, however, was not inhibited by z-VAD-fmk (Figure 5B). These results are consistent with previous results showing that translocation of cytochrome *c* from mitochondria is independent of caspase activation (21). To further investigate the potential role for cytochrome *c* in curcumin-induced apoptosis in Caki cells, we examined the effect of cyclosporin A, which inhibits cytochrome *c* release from the mitochondria, on curcumin-induced apoptosis. As shown in Figure 5C, cyclosporin A inhibits cytochrome *c* release from the mitochondria to the cytosol in curcumin-treated Caki cells. Activation of caspases by cytochrome *c* is a key event during apoptosis caused by various toxic agents and removal of serum growth factors. To confirm this result and determine whether caspase is activated after cytochrome *c*, we measured the change in caspase 3 activity in Caki cells after cyclosporin A. As shown in Figure 5D, caspase 3 activity markedly increases (~4-fold) in 75 μ M curcumin treated cells. However, co-treatment of Caki cells with 3 μ M cyclosporin A significantly reduces the rate of curcumin-induced caspase 3 activation.

Because Bax can undergo cleavage by activated caspases, we examined the effect of cyclosporin A on curcumin-induced cleavage of Bax protein. As shown in Figure 5C, curcumin-induced cleavage of Bax protein is partially blocked by cyclosporin A. The data clearly indicate that curcumin-induced apoptosis is associated with release of cytochrome *c* and caspase 3 activation.

Induction of apoptosis by curcumin appears to be dependent on formation of reactive metabolites

Many anti-neoplastic agents eliminate tumor cells by inducing programmed cell death or apoptosis, and numerous investigations have documented the cellular changes resulting from oxidative stress induced in cells following exposure to cytotoxic drugs and UV and γ irradiation (22,23). To examine the capacity of curcumin to cause intracellular oxidation, we used a specific fluorescent dye for H₂O₂, H₂DCFDA, which leads to an increase in fluorescent intensity when cells generate H₂O₂. As shown in Figure 6A, treatment with curcumin increases the H₂DCFDA-derived fluorescence. This increase in fluorescence is significantly inhibited by anti-oxidant, NAC. To examine the role of reactive oxygen species in curcumin-induced apoptosis, we used the antioxidant, NAC, which has

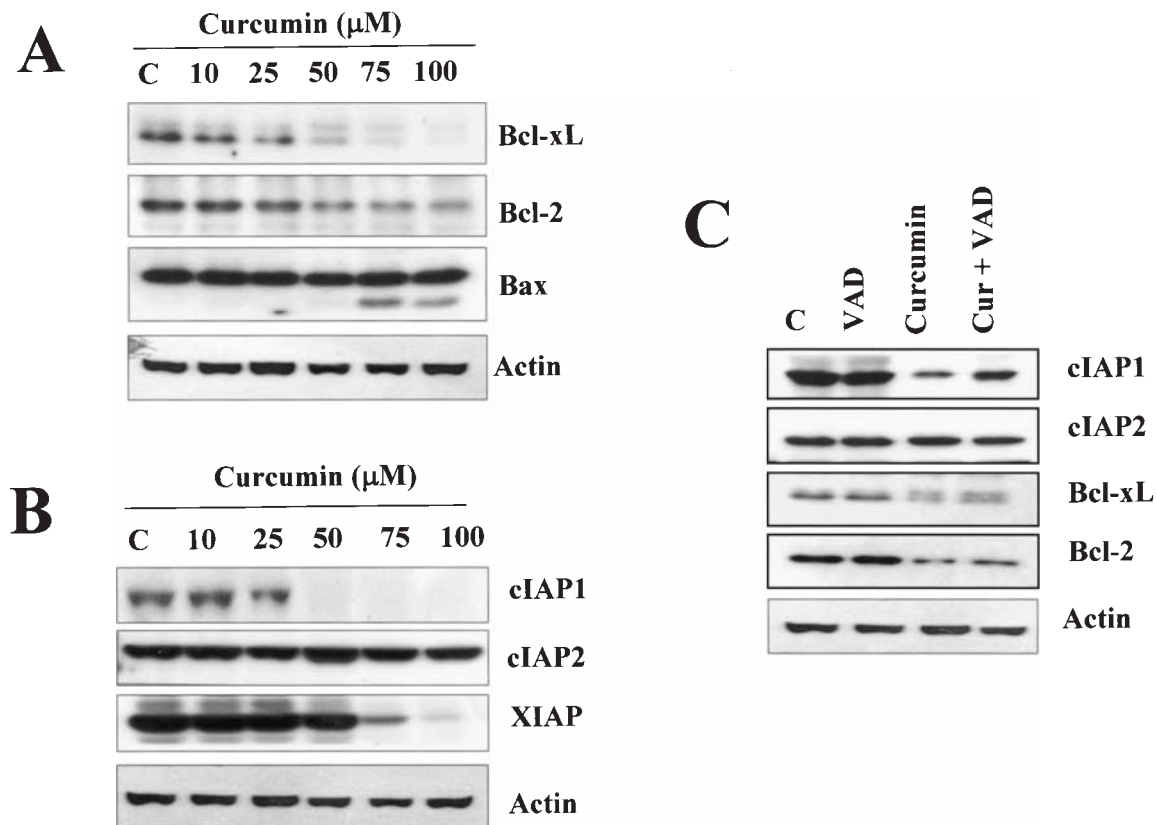


Fig. 4. The expression levels of apoptosis-related proteins in Caki cells by treatment with curcumin. (A) Caki cells were treated with indicated concentrations of curcumin. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-Bcl-2, anti-Bcl-X_L and anti-Bax). A representative study is shown; two additional experiments yielded similar results. (B) Caki cells were treated with indicated concentrations of curcumin. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-cIAP1, anti-cIAP2 and anti-XIAP). A representative study is shown; two additional experiments yielded similar results. (C) Effect of z-VAD-fmk on expression levels of apoptosis-related proteins. Caki cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with curcumin. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blot for cIAP1, cIAP2, Bcl-xL and Bcl-2.

been shown previously to be a thiol antioxidant that has functions as both a redox buffer and a reactive oxygen intermediate scavenger (24). Caki cells exposed to 10 mM NAC plus 75 μM curcumin for 24 h did not exhibit the characteristic features of cell shrinking, rounding, and partial detachment as cells exposed to curcumin alone. As shown in Figure 6B, co-treatment with NAC significantly prevents apoptotic morphological change.

Because prevention of apoptotic morphological change can be mediated in part by inhibition of caspase 3, we next evaluated the effect of NAC on caspase 3 activity. As shown in Figure 7A, caspase 3 activity markedly decreases in cells treated with NAC in addition to 75 μM curcumin. Furthermore, treatment with curcumin significantly decreases levels of pro-caspase 3, but has little effect on cells treated with NCA plus curcumin (Figure 7B). We also observed that NAC prevents cleavage of PLC- γ 1 in cells treated with NAC plus curcumin (Figure 7C). Because cytochrome *c* release is upstream of caspase 3 activation, we next examined whether NAC has a direct effect on curcumin-mediated cytochrome *c* release. Importantly, as shown in Figure 7D, NAC treatment of Caki cells significantly blocks curcumin-induced release of cytochrome *c* from the mitochondria into the cytoplasm. These data clearly indicate that NAC prevention of curcumin-induced apoptosis is associated with caspase inactivation and cytochrome *c* release.

Curcumin induces dephosphorylation of Akt in Caki cells

Previous studies have demonstrated that Akt activity inhibits the release of cytochrome *c* from mitochondria after UV irradiation (25). In addition, constitutively activated Akt significantly protected HMN1 cells from apoptosis induced by C2-ceramide (26). To determine whether curcumin-induced apoptosis is associated with Akt activity, we determined the expression and phosphorylation levels of Akt in Caki cells after treatment with various concentrations of curcumin. As shown in Figure 8A, the levels of phosphorylated Akt are significantly decreased in response to curcumin (75 μM). Phosphorylation of Akt is associated with activation of this kinase (27,28). As shown in Figure 8B, these effects of curcumin are time-dependent; Akt phosphorylation is rapidly decreased within 30 min of treatment, whereas total Akt protein levels remain constant during curcumin treatment.

To determine whether NAC prevents curcumin-mediated dephosphorylation of Akt, we measured phosphorylation levels of Akt in Caki cells after treatment with curcumin alone, or with NAC. As shown in Figure 8C, NAC treatment significantly blocks curcumin-induced dephosphorylation of Akt and release of cytochrome *c* from the mitochondria into the cytoplasm in Caki cells. The data clearly indicate that generation of ROS closely associated with curcumin-induced dephosphorylation of Akt, cytochrome *c* release and apoptosis. Interestingly, total Akt

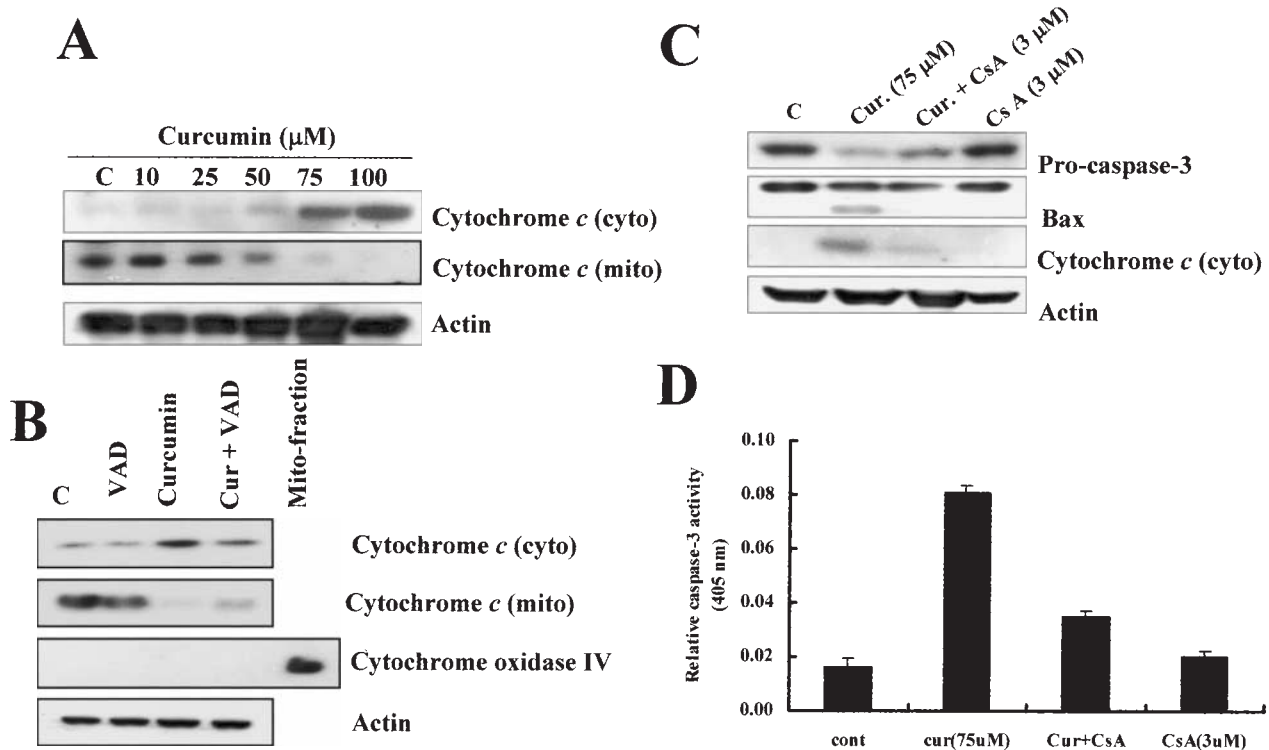


Fig. 5. Release of cytochrome *c* in curcumin-treated Caki cells. (A) Caki cells were treated with indicated concentrations of curcumin. Cytosolic extracts were prepared as described under Materials and methods. Thirty micrograms of cytosolic protein and mitochondrial fraction were resolved on 12% SDS-PAGE and then transferred to nitrocellulose, and probed with specific anti-cytochrome *c* antibody, or with anti-actin antibody to serve as control for the loading of protein level. (B) Effect of z-VAD-fmk on cytochrome *c* release. Caki cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with curcumin. Cytosolic and mitochondria extracts were prepared and subjected to SDS-PAGE followed by protein transfer. Immunoblot was performed with antibody against cytochrome *c* and cytochrome oxidase IV. (C) Effect of cyclosporin A on curcumin-induced cytochrome *c* release, expression levels of pro-caspase 3 and cleavage of Bax. Caki cells were incubated with cyclosporin A or solvent for 1 h before challenge with curcumin (75 μ M) for 20 h. For cytochrome *c* release, cytosolic extracts were prepared and analyzed as described. Equal amounts of cell lysates (40 μ g) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-caspase 3, anti-Bax and actin). Experiments were repeated three times. (D) Effect of cyclosporin A on curcumin-induced caspase 3 activation. Caki cells were incubated with cyclosporin A or solvent for 1 h before treatment with curcumin. Caspase activity was determined as described in Figure 2. Data shown are means \pm SD ($n = 3$).

protein levels are down-regulated by prolonged treatment with curcumin (Figure 8A and C).

In further studies of the relationship between total Akt protein and Akt mRNA in Caki cells, we measured Akt mRNA levels by RT-PCT. As shown in Figure 8D, Akt mRNA levels remain constant through the curcumin treatment period. The data suggest that curcumin-mediated down-regulation of total Akt protein is associated with post-transcriptional regulation. Akt protein is now known to be a target substrate of caspases (29). To address the possible role of caspase cleavage as a mechanism for down-regulation of Akt protein in curcumin-induced apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk. As shown in Figure 8E, curcumin treatment of Caki cells leads to down-regulation of Akt protein, and z-VAD-fmk pre-treated cells significantly attenuates this down-regulation of Akt protein. The data clearly indicate that curcumin-induced apoptosis is associated with Akt dephosphorylation and down-regulation of Akt protein.

Recently, the Bcl-2 family member, Bad, has been suggested to mediate the anti-apoptotic effects of the Akt pathway (30). We reasoned that if Bad is a downstream effector of Akt, then inhibition of Akt activity by curcumin should also result in inhibition of Bad phosphorylation. As shown in Figure 8F, curcumin does not inhibit Bad phosphorylation. These results indicate that curcumin-induced apoptosis may not correlate with Bad phosphorylation in our systems.

Discussion

Our results show that curcumin induces apoptosis in Caki cells. At the apoptosis-inducing concentrations, curcumin also stimulates dephosphorylation of Akt, proteolytic activities of caspase 3 and release of cytochrome *c*. Inhibition of caspase activation by z-VAD-fmk or cytochrome *c* release by cyclosporin A attenuates curcumin-induced apoptosis. In addition, NAC, which is an antioxidant, inhibits curcumin-mediated apoptosis through inhibition of caspase activation and cytochrome *c* release. Thus, our experiments indicate that curcumin-induced apoptosis in Caki cells is mediated by oxidants, the release of mitochondrial cytochrome *c*, and it is the subsequent activation of caspase 3. An understanding of the factors that regulate the cellular response to ROS and the molecular mechanisms by which they interact with cellular constituents, as well as the consequences of such interactions, are important fundamental goals of biomedical research. Renal cells are particularly sensitive to oxidant-mediated injury.

Our finding of curcumin-induced translocation of cytochrome *c* from the mitochondria to the cytosol provides a direct link between the mitochondria and curcumin-induced apoptosis in renal cells, Caki cells. The role of cytochrome *c* release in curcumin-induced apoptosis is further supported by observations that cyclosporin A blocks this series of events in Caki cells. Cytochrome *c* appears to be a critical factor in this

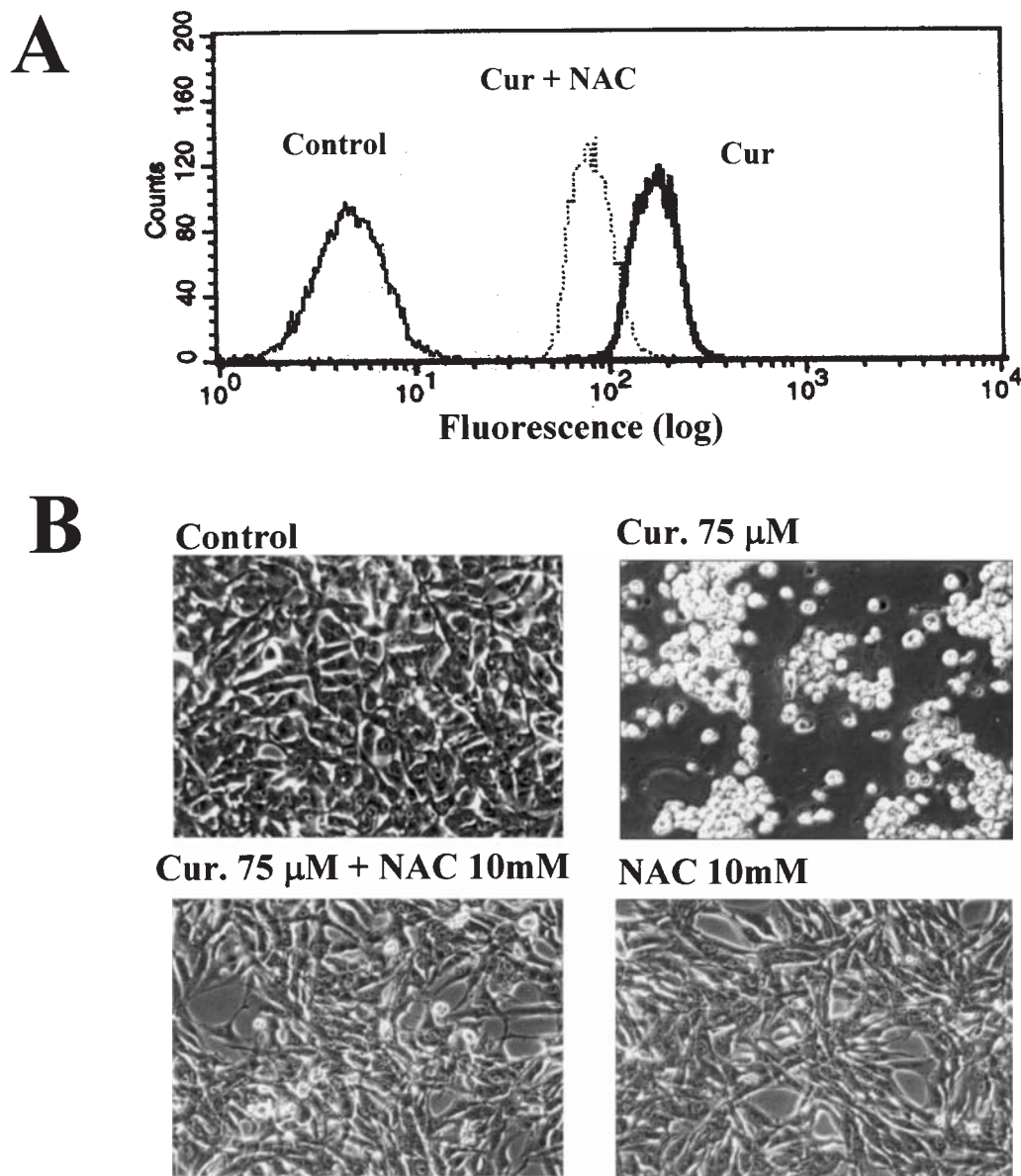


Fig. 6. Effects of NAC on curcumin-induced ROS generation and morphological characteristics of Caki cells. (A) To determine the intracellular content of peroxides, Caki cells were loaded with H2DCFDA, and the fluorescence was measured by flow cytometry. (B) Caki cells were treated with vehicle, 75 μ M curcumin, 75 μ M curcumin plus 10 mM NAC, and 10 mM NAC for 24 h. Magnification, $\times 200$.

process, directly activating caspases by binding to Apaf-1 in the presence of ATP (31).

Members of the Bcl-2 family of proteins have been demonstrated to be associated with the mitochondrial membrane and regulate its integrity (32). Our finding that Bcl-2 protein decreases in response to curcumin suggests that the Bcl-2 protein may play a key role in curcumin-induced apoptosis of Caki cells, although the mechanisms underlying curcumin-mediated reduction of Bcl-2 are not clear at present. In the present study, we found that unlike Bcl-2, curcumin treatment does not result in a change in Bax protein levels. Bax exerts proapoptotic activity by translocation from the cytosol to the mitochondria, where it induces cytochrome *c* release, whereas Bcl-2 exerts its anti-apoptotic activity, at least in part, by inhibiting the translocation of Bax to the mitochondria (33,34). By down-regulating Bcl-2 levels in Caki cells,

curcumin may promote the translocation of Bax from the cytosol to the mitochondrial membrane, leading to the release of cytochrome *c*.

Our data show that the release of cytochrome *c* from mitochondria in Caki cells precedes caspase 3 activation by curcumin. Another factor contributing to caspase 3 activation in curcumin treated Caki cells may be decreased IAP expression. Human IAP proteins, including XIAP, c-IAP1, c-IAP2, NAIP and survivin, are characterized by the presence of one to three copies of a 70 amino acid motif, the baculoviral inhibitory repeat domain, which bears homology to sequences found in the baculovirus IAP proteins (35). IAPs have been reported to inhibit apoptosis due to their function as direct inhibitors of activated effector caspases, caspase 3 and caspase 7. Furthermore, cIAP1 and cIAP2 are also able to inhibit cytochrome *c*-induced activation of caspase 9 (36,37).

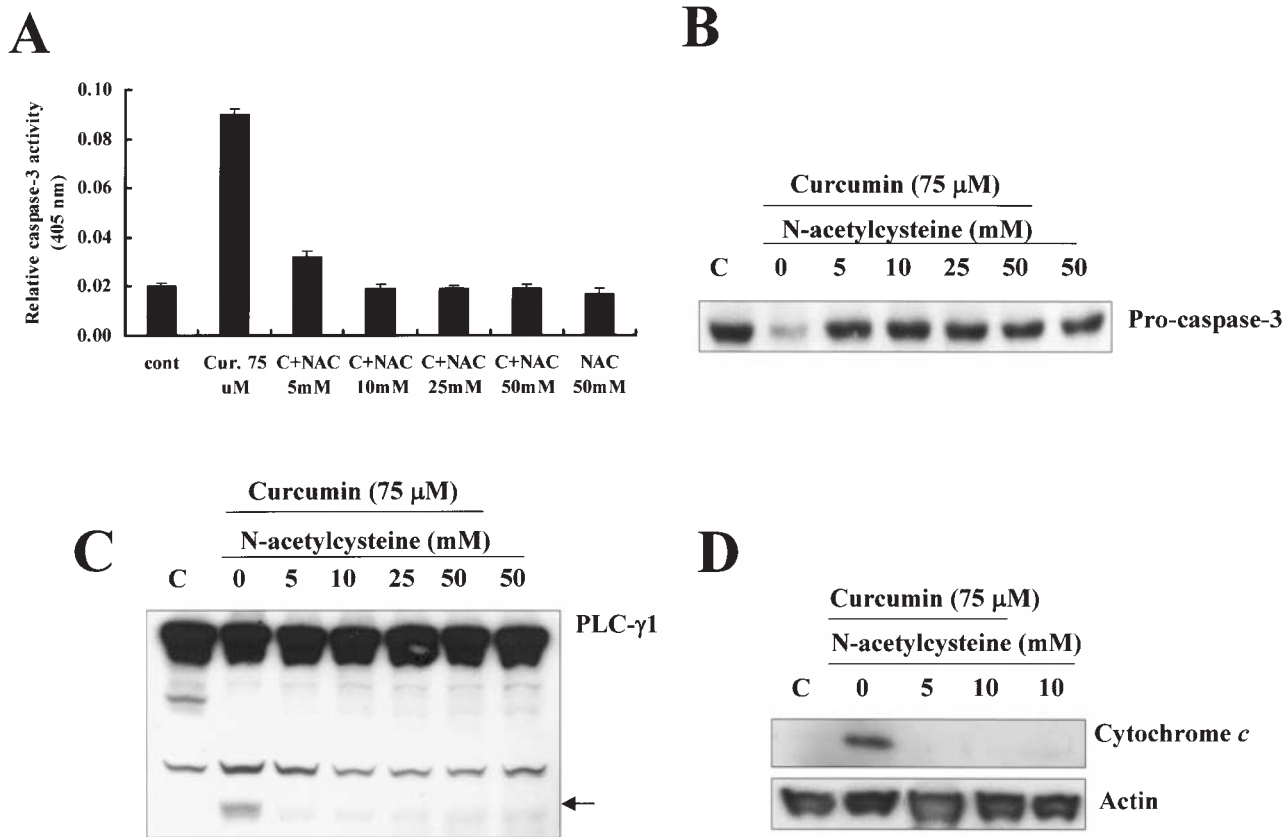


Fig. 7. Effect of NAC on curcumin-induced caspase 3 activity and cytochrome *c* release. (A) Caki cells were incubated with indicated concentration of NAC or solvent for 1 h before challenge with curcumin (75 μ M) for 20 h. Caspase 3 activity was determined as described in Figure 2. Data shown are means \pm SD ($n = 3$). (B) Equal amounts of cell lysates (40 μ g) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-caspase 3 antibodies. (C) Cells were treated with the indicated concentrations of NAC. Equal amounts of cell lysates (40 μ g) were subjected to electrophoresis and analyzed by western blot for PLC- γ 1. The proteolytic cleavage of PLC- γ 1 is indicated by an arrow. (D) Thirty micrograms of cytosolic protein was resolved on 12 % SDS-PAGE and then transferred to nitrocellulose, and probed with specific anti-cytochrome *c* antibody, or with anti-actin antibody to serve as control for the loading of protein level.

Recently it has been suggested that oxidative stress plays a role as a common mediator of apoptosis (38). We report here that curcumin induces oxidative stress and apoptosis in Caki cells, and that the antioxidant NAC prevents apoptosis induced by curcumin. Our data also show that the antioxidant NAC prevents caspase 3 activation, dephosphorylation of Akt and cytochrome *c* release. Thus, curcumin causes oxidative stress, which then leads to the release of cytochrome *c* from mitochondria, further initiating the activation of the execution caspases and leading to apoptotic cell death. Further, the down-regulation of Bcl-2 and IAP family proteins may also lead to the activation of caspase 3.

In addition, NAC induces activation of Akt by increasing the level of phosphorylated active Akt. Akt is a protein kinase that promotes cell survival by inhibiting apoptosis, although precisely how it exerts its anti-apoptotic effects is not clear. It has recently been reported to directly phosphorylate and inactivate procaspase-9 as well as to block caspase-9-mediated apoptosis induced by cytochrome *c* via Apaf-1 in response to an early apoptotic stimuli (39). Akt can also phosphorylate Bad, resulting in the release of Bcl-2 and inhibition of the apoptotic process (30).

In contrast with our results, it has been reported that curcumin induces caspase-3-independent apoptosis in human multi-drug-resistant cells (40). Moreover, it was demonstrated in Jurkat cells that curcumin induced non-typical apoptosis-like pathway, which was independent of mitochondria and

caspase-3 (41). In addition, there are conflicting results regarding its apoptogenic mechanism in other cell types. Piwocka *et al.* (42) showed that curcumin induced apoptosis through the depletion of glutathione in Jurkat cells, whereas Jaruga *et al.* (43) reported that curcumin prevent dexamethasone-induced apoptosis via induction of glutathione synthesis in thymocytes.

Until now, mechanism for curcumin-induced apoptosis is not yet fully defined. Although curcumin was shown to induce apoptosis of a number of human cancer cell lines, little information is available regarding how curcumin induces apoptosis in renal cells. In the present study, we demonstrate novel findings that in renal carcinoma cells, curcumin has several different molecular targets including inactivation of Akt signalling pathways that could contribute to inhibition of proliferation and induction of apoptosis, down-regulation of anti-apoptotic proteins, and release of cytochrome *c*, activation of caspase-3. Curcumin is world widely used as a coloring and flavoring additive in many foods and has attracted interest because of its anti-inflammatory and chemopreventive activities. It has been recently reported that the curcumin required for efficacy of chemoprevention in humans would be a daily dose of 1.6 g/person without adverse effect (44). It is pharmacokinetically non-permissible to take the human blood volume and calculate concentration of drug on the basis of the dose ingested. Clinical trials of oral curcumin cannot be utilized because of low bioavailability or low target organ concentration due to its rapid metabolism in liver and intestinal wall.

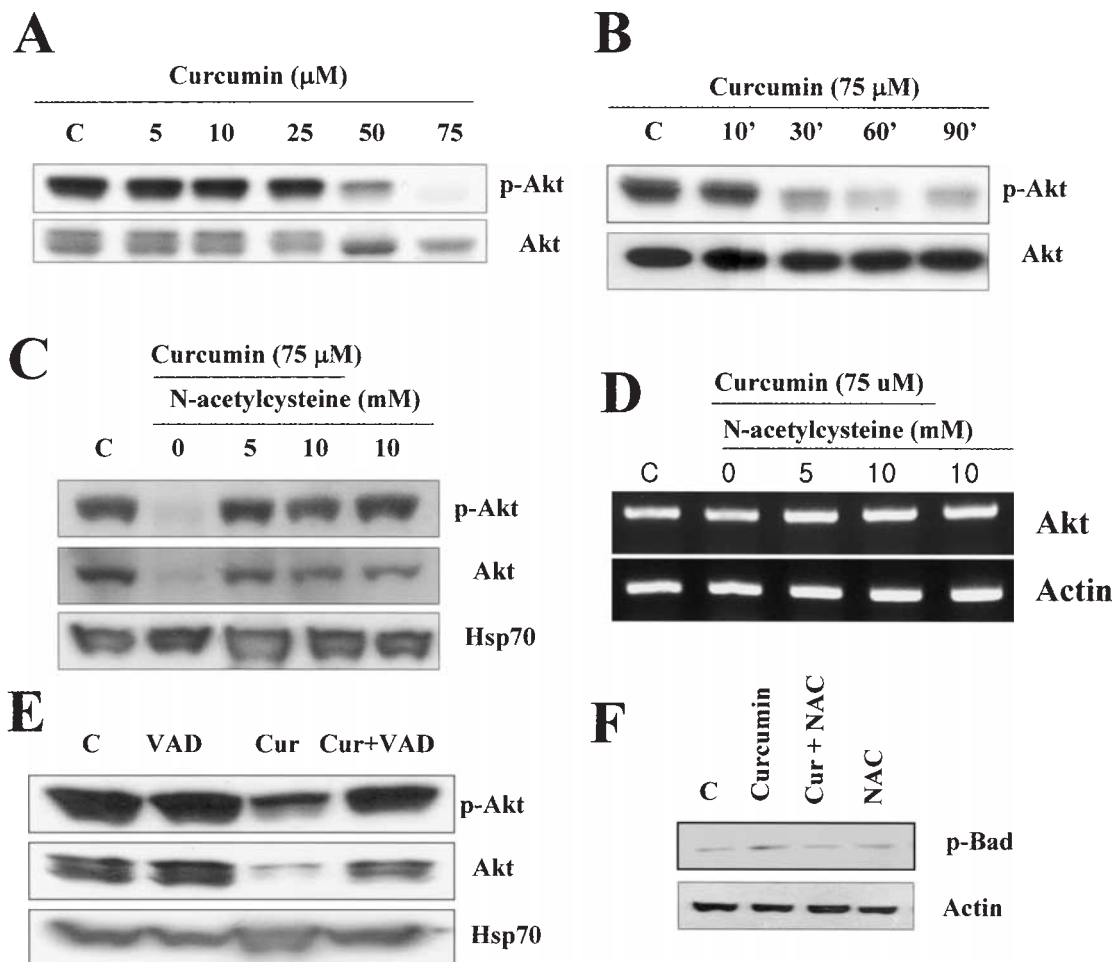


Fig. 8. Inactivation of Akt in curcumin-treated Caki cells. (A) Caki cells were treated with indicated concentrations of curcumin. Equal amounts of cell lysates (40 μ g) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific phosphorylated Akt antibodies and anti-Akt antibodies. A representative study is shown; two additional experiments yielded similar results. (B) Whole cell lysates, prepared from curcumin-treated Caki cells for different times, were subjected to immunoblot analysis using specific phosphorylated Akt antibodies and anti-Akt antibodies. (C) Effect of NAC on curcumin-induced dephosphorylation of Akt and cytochrome *c* release. Caki cells were incubated with the indicated concentration of NAC or solvent for 1 h before challenge with curcumin (75 μ M) for 20 h. Equal amounts of cell lysates (40 μ g) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific phosphorylated Akt antibodies and anti-Akt antibodies. (D) Akt mRNA expression levels by curcumin treatment. Caki cells were incubated with the indicated concentration of NAC or solvent for 1 h before challenge with curcumin (75 μ M) for 20 h and RT-PCR analysis was performed. (E) Effect of z-VAD-fmk on Akt expression levels. Caki cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with curcumin. Equal amounts of cell lysates (40 μ g) were subjected to electrophoresis and analyzed by western blot for p-Akt and Akt. (F) Effect of z-VAD-fmk on Bad phosphorylation levels. Caki cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with curcumin. Equal amounts of cell lysates (40 μ g) were subjected to electrophoresis and analyzed by western blot for p-Bad and actin.

However, co-ingestion of curcumin with the pepper constituent l-piperoylpiperidine, which is thought to inhibit xenobiotic glucuronidation, appeared to increase curcumin serum AUC by a factor of 20 (45). In addition, a recent clinical pilot study in colorectal cancer suggests that *Curcuma* extract can be administered safely to patients at doses of up to 2.2 g daily, equivalent to 180 mg of curcumin (46).

In summary, our studies demonstrate that curcumin treatment of Caki cells induces cytochrome *c* release, which activates pro-caspase 3 and DNA fragmentation. Moreover, antioxidant NAC inhibits curcumin-induced apoptosis and prevents inactivation of Akt and the release of cytochrome *c* from the mitochondria. Down-regulation of the Bcl-2 and IAP proteins, therefore, maintains caspase 3 in the active state and stimulates the molecular cascade of apoptosis. In view of accumulating evidences that curcumin may be an important determinant of clinical response in cancer, further efforts to explore this therapeutic strategy appear warranted.

Acknowledgements

This work was supported by a grant (01-PJ1-PG3-208000-0026) of the Korea Health 21 R&D project, Ministry of Health and Welfare and partially supported by grant No R13-2002-028-01002-0 from the MRC Program of the Korea Science & Engineering Foundation.

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Received December 17, 2002; revised April 25, 2003;
accepted April 28, 2003