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GADD153 mediated curcumin induced cell cycle arrest and apoptosis in human lung carcinoma A-549 Cells through the elevation of Ca^{2+} productions, decreasing of mitochondrial membrane potential and promoted caspase-3 activation

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Abstract

Curcumin, a major component of the Curcuma species, is known to have antioxidant, anti-inflammatory properties and can induce cancer cells' apoptosis. However, the precise molecular mechanisms of these effects on apoptosis *in vitro* are unclear. In this study, we showed that curcumin, a plant product containing the phenolic phytochemical, is a potent enhancer of Gadd153, and can cause mitochondria-dependent-induced apoptosis through the activation caspase-3 at treatment concentration of 30 μM in human lung cancer A-549 cells. In contrast, treatment with 5-10 μM of curcumin does not induce significant apoptosis, but rather triggers induced G2/M phase arrest in A-549 cells. We use flow cytometric analysis which indicated that curcumin directly increased intracellular oxidative stress based on the cell permeable dye 20,70-dichlorofluorescin diacetate (DCFH-DA) acting as an indicator of reactive oxygen species (ROS) generation. Using the dye Indo-1/AM as an indicator of Ca^{2+} generation, it showed that curcumin increase the levels of Ca^{2+} from ER. Using the dye DEioC6 as an indicator of mitochondrial membrane potential ($\Delta\Psi_m$), it demonstrated that curcumin decreased the levels of $\Delta\Psi_m$ in A-549 cells. The result from Western blotting demonstrated that curcumin induced apoptosis from mitochondria-dependent through the activation of caspase 3- activation. Ca^{2+} also plays important role in the apoptosis pathway in curcumin treated cells because the A-549 cells were pretreated with the Ca^{2+} chelator (BAPTA) before leading to decreasing the apoptosis. Curcumin induced the production of ROS which caused ER stress based on the gene expression of Gadd153 from A-549 cells. Overall, our results demonstrated that curcumin dosage treatment determines the possible effect on ROS generation, ER stress, Ca^{2+} levels, $\Delta\Psi_m$ levels, caspase-3 activation and cell apoptosis in A-549 cells.

Keywords: Curcumin; reactive oxygen species (ROS), Ca^{2+}, mitochondrial membrane potential ($\Delta\Psi_m$), caspase-3, cell cycle arrest, apoptosis

1. Introduction

Cell cycle is a series of tightly integrated events which allow the cell to grow, proliferate and die, and it serves to protect the cell from genotoxic stress. Cell cycle can be divided into G0/G1-, S- and G2/M- phase based on the DNA content. The cyclins, cyclin-dependent kinases (CDKs) and even some of the inhibitors of cyclins and CDKs are involved in cell cycle. CDKs promoted the cell to move from one phase to the next pharse (G0/G1 to S, S to G2/M or G2 to M) of the cell cycle. If agents affect the cyclin and/or CDKs, it may lead to cell cycle arrest. If a cell continues to cycle with its damaged DNA intact, the apoptotic machinery is triggered and the cell will undergo apoptosis.

Numerous naturally occurring substances are recognized to be an antioxidants and cancer preventive agents or even as a cancer therapy drug such as taxol [1]. Those bioactive substances exert their anti-cancer activity through blocking cell cycle progression and triggering tumor cell apoptosis. Therefore, the best strategy for anticancer activity of agents is causing cell cycle arrest and the induction of apoptosis in tumor cells [2, 3].

Curcumin (diferuloylmethane), a phenolic compound from the plant Curcuma longa (Linn), is a widely used flavoring agent in food [4]. Curcumin inhibits cell proliferation and induces apoptosis in human leukemia, prostate cancer, and non-small cell lung cancer cell lines [5-7]. Curcumin has been shown to have antitumor activity in the colon, skin, stomach, duodenum, soft palate, and breasts of

rodents [8-10]. Recently published phase I clinical trial of oral curcumin in colon cancer patients showed high tolerability of the drug in humans and proposed a possibility of developing curcumin as an oral cancer preventive or therapeutic agent [11]. TNF and/or cigarette smoke were shown to activate NF- kappaB in human non-small cell lung cancer cells by phosphorylation and degradation of IkBalpha and the translocation of p65 subunit [12]. The anti-tumor activity of curcumin is attributed to its ability to induce apoptosis via caspase-3 activation [13].

Although there have been extensive investigations to elucidate the mechanism of action of curcumin as an antitumor agent, the exact mechanism is yet to be established. Therefore, in the present study, we focused on the effect of curcumin on lung cancer cells and also investigated the exact mechanism of curcumin induced apoptosis in human lung cancer A549 cells.

2. Materials and Methods

2.1 Chemical and reagents

Curcumin, propidium iodide (PI), triton X-100, Tris-HCl, trypan blue, ribonuclease-A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium phosphates, dimethyl sulfoxide (DMSO), and TE buffer were purchased from Merck Co. (Darmstadt, Germany). 90% Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate + 10% fetal bovine serum, penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

2.2 Human lung carcinoma A-549 cell line

Human lung carcinoma A-549 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The A-549 cells were placed into 75 cm^3 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 atmosphere in 90% Ham's F12K medium with 2 mM L-glutamine ajusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY), and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) [14].

2.3 Morphological changes and cell viability of A-549 cells treated with curcumin were determined by using pharse-contrast microscope and flow cytometry

The A-549 cells were plated in 12-well plates at a density of $2x10^5$ cells/well and grown for 24 h. Different concentrations of curcumin were then added to cells for final concentration 0, 5, 10, 20, 30 and 50 μM, while only adding DMSO (solvent) for the control regimen and grown at 37°C, 5% CO_2 and 95% air for a different period of time. To determine morphological changes and cell viability, the pharse-contrast microscope and the flow cytometric assay was used as described previously [14, 15].

2.4 Cell cycle and apoptosis of A-549 cells treated with different concentrations of curcumin were examined by using Flow cytometry analysis.

Approximately $5x10^5$ cells/well of A-549 cells in 12-well plate with various concentrations (0, 5, 10, 20 30 and 50 μM, respectively) of curcumin were incubated in an incubator for different time periods. Cells were harvested by centrifugation

and were fixed gently (drop by drop) by putting 70% ethanol (in PBS) in 4°C overnight and then re-suspended in PBS containing 40 μg/mL PI and 0.1 mg/mL RNase and 0.1% Triton X-100 in dark room. After 30 minutes at 37°C, the cells were analyzed with a flow-cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488 nm wavelength. Then the cell cycle was determined and analyzed [13]. Annexin V-FITC and PI double staining kit from PharMingen (San Diego, CA) were used for apoptotic cells quantified [14-16].

2.5 Apoptosis of A-549 cells treated with different concentrations of curcumin were examined by using DAPI staining

Approximately $2x10^5$ cells/well of A-549 cells in 12-well plate with 30 μM curcumin were incubated in an incubator for 48 hours. Cells were stained by DAPI, then photographed by fluorescence microscope as described previously [14-16].

2.6. Comet assay examining the curcumin-induced DNA damage of A-549 cells.

Approximately $5x10^3$ cells/ml cells of A-549 were treated with or without various concentrations of curcumin at 0, 5, 10, 20, 30 and 50 μM for 24 hours then isolated for examining DNA damage by using the Comet assay described previously [14].

2.7 Caspase-3 activity determination of A-549 cells treated with curcumin and/or cells were pretreated with caspase-3 inhibitor (Ac-DEVD-CHO) then with curcumin.

Approximately $5x10^5$ cells/well of A-549 cells in 12-well plate with various concentrations (0, 5, 10, 20 30 and 50 μM) of curcumin were incubated in an incubator for different time periods. Cells were harvested by centrifugation and the mediums were removed. Then, 50 μL of 10 μM substrate solution (PhiPhilux is a unique class of substrates for caspase-3) was added to the cell pellet ($1x10^5$ cells per

sample) or cells were pretreated with caspase-3 inhibitor (Ac-DEVD-CHO) for 3 hours, then treated with 30 μM curcumin. Cells then were incubated at 37°C for 60 minutes. Then the cells were washed once by adding 1 mL of ice cold PBS and re-suspend in fresh 1 mL. Cells were analyzed with a flow-cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argonion laser at 488 nm wavelength. Then the caspase-3 activity was determined and analyzed [14]. The MMP and apoptosis were determinated [17].

2.8. Detection of reactive oxygen species (ROS) in A-549 cells after treated with curcumin by flow cytometry

Approximately $5x10^5$ cells/well of A-549 cells in 12-well plate with various 30 μM curcumin were incubated for 15, 30, 45, 60 and 120 minutes to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 μL of 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) and incubated at 37°C for 30 min and analyzed by flow cytometry [14-16].

2.9. Detection of mitochondrial membrane potential ($\Delta\Psi_m$) in A-549 cells after treated with curcumin by flow cytometry

Approximately $5x10^5$ cells/well of A-549 cells in 12-well plate with various concentrations (0, 5, 10, 20, 30 or 40 μM, respectively) of curcumin were incubated for 24 h to detect the changes of $\Delta\Psi_m$. The cells were harvested and washed twice, re-suspended in 500 μL of $DiOC_6$ (4 mol/L) and incubated at 37°C for 30 min and analyzed by flow cytometry [14-16].

2.10. Detection of Ca^{2+} concentrations in A-549 cells after treated with curcumin by flow cytometry

Approximately $2x10^5$ cells/well of A-549 cells in 12-well plate with 30 μM curcumin were incubated for 6, 12, 24 and 48 h to detect the changes of Ca^{2+} concentration. The cells were harvested and washed twice, once for apoptosis analysis and the other underwent re-suspendion in Indo 1/AM (3 μg/ml) and was incubated at 37°C for 30 min and analyzed by flow cytometry [14-16].

2.11. Detection of Ca^{2+} concentrations and MMP levels and apoptosis in A-549 cells after pre-treated with BAPTA then treated with curcumin.

The level of Ca^{2+}, $\Delta\Psi_m$ and apoptosis of the A-549 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the Indo 1/AM and $DiOC_6$ (Calbiochem; La Jolla, CA) and PI, respectively. About $5x10^5$ cells/well of A-549 cells in 12-well plate were pre-treated with BAPTA before adding 30 μM of curcumin for incubation for 24 h to detect the changes of Ca^{+2} concentration and the levels of $\Delta\Psi_m$ and percentage of apoptosis. The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/ml) and $DiOC_6$ (4 mol/L) before being incubated at 37°C for 30 min and analyzed by flow cytometry [15-16].

2.12. Curcumin affected the expressions of Wee1, CHK1, Cdc25c, p53, p21, Cdc2, Cyclin B1,cspase-3 and -9, Bcl-2, COX-1 and -2, Naf-kB, catalase, SOD(Zn and Cu), MMP-2 and -9 in A-549 cells.

The total proteins were collected from A-549 cells treated with 30 μM for 0, 6, 12, 24 and 48 h before Wee1, CHK1, Cdc25c, p53, p21, Cdc2, Cyclin B1,cspase-3 and -9, Bcl-2, COX-1 and -2, Naf-kB, catalase, SOD(Zn and Cu), MMP-2 and -9 were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously [14-17].

2.13. Curcumin affects the levels of Gadd153 mRNA in A-549 cells.

The total RNA was extracted from the A-549 cells after treatment 30 μM curcumin for 1, 3, 6 and 12 h, using the Qiagen RNeasy Mini Kit as described previously [18-20]. The PCR reactions were performed with the following primers, as described previously (Chung et al., 2001, Tang et al., 2006). The sequences of the primers were: Gadd153: 5V-GAAACGGAAACAGAGTGGTCATTCCCC-3V (sense) and 5V-GTGGGATTGAGGGTCACATCATTGGCA-3V (antisense) to produce a 301-bp DNA fragment of Gadd153. In each PCR, an internal control using the primers was: GAPDH: 5V-CTCAGACACCATGGGGAAGGTGA-3V (sense) and 5V-ATGATCTTGAGGCTGTTGTCATA-3V (antisense) to produce a 450-bp fragment of the GAPDH gene that was included. Each assay was conducted at least twice to ensure reproducibility [18-20].

2.14. Curcumin affects the migration of A-549 cells.

The $2x10^5$ cells/well of A-549 cells in 10 cm-plate with 30 μM curcumin were incubated for 12 and 21 h to detect the migration of A-549 cell as described previously [14] .

2.15. Statistical analysis

Student t-test was used to analyze the statistical analysis between the capsaicin treated and control groups.

3. Results

3.1 Ccurcumin induced morphological changes and decreased the percentage of viable A-549 cells.

After A-549 cells were treated with or without various concentrations of curcumin for various time periods, morphological changes were photographed by using pharse-contrast microscope (Fig. 1A) and viable cells were examined by flow cytometric methods. The results demonstrated that curcumin induced morphological changes (Fig. 1A) and decreased the percentage of viable cells (Fig. 1B) and is of a time-dependent (Fig. 1A) and dose-dependent (Fig. 1B) manner.

3.2 Curcumin induced cell cycle arrest and apoptosis in A-549 cells.

A-549 cells were treated with or without various concentrations of curcumin for various time periods before cell cycle and apoptosis (sub-G1 group) were examined and analyzed by flow cytometric methods. The results demonstrated that curcumin induced G2/M phase arrest (Fig. 2A) (dose-dependent effects) and apoptosis (Fig. 2B) (time-dependent manner).

3.3 Effects of curcumin on induction of apoptosis on A-549 cells were examined by

DAPI staining.

Induction of apoptosis from A-549 cells after treated with curcumin that were determined. Apoptosis was detected by DAPI staining method after 6, 12, 24 and 48 hours of continuous exposure to curcumin before being photographed by fluorescence microscope. As shown in Figure 3, curcumin induced apoptosis in a time-dependent manner.

3.4. Curcumin-induced DNA damage in A-549 cells was examined by Comet assay.

The Comet assay examining showed that curcumin-induced DNA damage in A-549 cells based on the smear DNA migration. The higher concentrations of curcumin led to longer DNA migration smear (Fig. 4).

3.5 *Curcumin promoted the caspase-3 activity and caspase-3 inhibitor (Ac-DEVD-CHO) inhibits caspase-3 activity in A-549 cells.*

After A-549 cells were pre-treated with or without caspase-3 inhibitor (Ac-DEVD-CHO) they were then treated with various concentrations of curaumin for 24 h before caspase-3 activity was analyzed by flow cytometric methods. The results demonstrated that curcumin promoted the activation of caspase-3 (Fig. 5A) and those effects are dose-dependent manner. The inhibitor of caspase-3 (Ac-DEVD-CHO) decreased the levels of MMP (Fig. 5B) and percentage of apoptosis (Fig. 5C).

3.6. Curcumin induced the production of reactive oxygen species (ROS) in A-549

cells.

After A-549 cells were treated with or without 30 μM curcumin for various time periods, ROS productions were analyzed and quantitated by flow cytometric methods. The results demonstrated that curcumin promoted the levels of ROS and those effects are time-dependent manner (Fig 6).

3.7 Curcumin suppressed the levels of mitochondria membrane potential ($\Delta\Psi_m$) inA-549 cells.

After A-549 cells were treated with various concentrations of curcumin for 12 h, the levels of $\Delta\Psi_m$ were analyzed and quantitied by flow cytometric methods. The results demonstrated that curcumin increased in the loss of mitochondrial $\Delta\Psi_m$ and those effects are dose-dependent manner (Fig. 7).

3.8. Curcumin induced the Ca^{2+} production in A-549 cells.

After A-549 cells were treated with or without 30 μM curcumin for 6, 12, 24 and 48 h, Ca^{2+} productions were analyzed by flow cytometric methods. The results demonstrated that curcumin promoted the Ca^{2+} productions and those effects are time-dependent manner (Fig. 8).

3.9. *Effects of calcium antagonist BAPTA (Ca^{2+} chelator) on curcumin affect the levels of Ca^{2+}, MMP and apoptosis in A-549 cells*

After A-549 cells were pre-treated with BAPTA (Ca^{2+} chelator), they were then

treated with 30 μM curcumin for 24 h, while Ca^{2+} productions, levels of MMP and apoptosis were analyzed by flow cytometric methods. The results demonstrated that BAPTA decreased curcumin-induced Ca^{2+} productions (Fig. 9A), decreased the levels of MMP (Fig. 9B) and also decreased the percentage of apoptosis (Fig. 9C).

3.10. Curcumin affected the expressions of Wee1, CHK1, Cdc25c, p53, p21, Cdc2, Cyclin B1,cspase-3 and -9, Bcl-2, COX-1 and -2, Naf-kB, catalase, SOD(Zn and Cu), MMP-2 and -9 in A-549 cells.

In order to characterize the molecular mechanism of curcumin-induced cell cycle arrest and apoptosis in A-549 cells, we examined the expressions of cell cycle- and apoptosis-associated proteins during the treatment with curcumin by Western blotting. The protein levels of Wee1, CHK1, Cdc25c, p53, p21, Cdc2, Cyclin B1 (Fig. 10A), caspase-3 and-9 (Fig. 10B), Bcl-2, COX-1 and -2, Naf-kB, catalase, SOD(Zn and Cu) (Fig. 10C), MMP-2 and-9 (Fig. 10D) from Western blot indicate that the levels of CHK1,CDC25c, CDC2, cyclin B1, Bcl-2, NF-kaapB, catalase, MMP-2 and-9 that were decreased and the levels of Wee1, p53, p21, caspase-3 and-9, COX-2 that were increased by curcumin may have led to G2/M arrests and apoptosis.

3.11. Curcumin inhibited Gadd153mRNA expression in A-549 cells.

The results from PCR indicated that curcumin treated A-549 cells led to the levels of mRNA Gadd153 increased (Fig 11A and B). These effects are time-dependent manner.

3.13. Curcumin affects the migration of A-549 cells.

The results from pharse-contrast microscope showed that curcumin inhibited the migration of A-549 cells and these effects are time-dependent (Fig. 12).

4. Discussion

Although many studies had demonstrated that curcumin induced cell cycle arrest and apoptosis in human cancer cell lines, the exact role of Gadd153 on the induction of apoptosis by curcumin is still unclear. Therefore, in the present study we focused on the role of Gadd153 associated apoptosis which from human lung cancer A-549 cells after exposed to curcumin. Overall our results showed that curcumin induced G2/M arrest and apoptosis in A-549 cells and this is in agreement with other reports that demonstrated that curcumin induces G2/M phase arrest and apoptosis in examined human melanoma cells [21], bladder cancer T24 cells [22] and gastric and colon cancer cells [23]. Our data also showed that curcumin induced apoptosis through decreasing the levels of $\Delta\Psi_m$, cytochrome c release and promoted caspase-3 activation. This is in agreement with other reports which showed that curcumin induced apoptosis through the activation of caspase-3 [23].

It is well known that normal cells maintain the cell in cell cycle and the cell cycle checkpoints are important control mechanisms that ensure the proper execution of cell cycle events. CDKs are the major molecular players with cyclins in cell cycle progression [24]. If agent inhibits the CDK activity or affects the levels of CDK, it could cause cell cycle arrest that had been demonstrated to be the important strategy for the potential of novel anticancer drugs [24]. CDK inhibitors (flavopiridol, UCN-01, olomoucine, rocosvitine, butyrolactone I, indirubin-5-sulfonate, and indirubin-3′-monoxime) may inhibit the growth of cancer cells by causing cell cycle

arrest and apoptosis [24]. Those agents can induce DNA damage and if the CDKs are inhibited, both could lead to the cell undergoing cell-cycle arrest that may provide the cell the opportunity to repair its own damaged DNA before it resumes cell proliferation. Furthermore, if the cells continue to cycle with its damaged DNA intact, it may trigger the apoptotic machinery and the cell will undergo apoptosis. Our results also demonstrated that curcumin induced DNA damage based on the Comet assay as showed in Figure 4. Promote apoptosis of DNA damaged tumor cells could be performed either during cell cycle arrest or following premature cell cycle checkpoint exit that tumor cells re-enter the cell cycle before DNA repair is complete [24].

Our data also showed that curcumin induced ROS production, Ca^{2+} release and decreased the levels of $\Delta\Psi_m$ and promoted the cytochrome c release from mitochondria then followed by activation of caspase-3 before apoptosis occur. High levels of ROS not only induced cell death [25] but also cause DNA damage and genomic instability [26, 27] that may cause tumorigenesis. The reason is that in certain tumor cells, there exists higher levels of ROS [28] but low levels of ROS enhance cell proliferation [29]. We also found that curcumin induced the levels of Gadd153 in earlier treated time (Fig. 11). So it may be that curcumin induced ROS production followed by ER stress and DNA damage which caused Ca^{2+} released then also promoted p53 levels leading to Bax increasing and decreasing the levels of Bcl-2 followed by decreased levels of $\Delta\Psi_m$ in mitochondria (Fig. 13). Much evidence had showed that mitochondrion plays an important role in the regulation of apoptosis [30, 31]. Mitochondrial dysfunctions including the loss of mitochondrial membrane potential ($\Delta\Psi_m$), permeability transition, and release of cytochrome *c* from the mitochondrion into the cytosol are associated with apoptosis [32]. It is also reported that agents induced apoptosis also promoted Gadd153 levels in earlier time [33].

We also found out that A-549 cells pretreated with BAPTA (a chelator of Ca^{2+}) then followed by treatment with curcumin could decrease the percentage of apoptosis which was induced by curcumin. The result also showed BAPTA (a celator of Ca^{2+}) could decrease the levels of $\Delta\Psi_m$ in examined cells. Apparently Ca^{2+} was involved in curcumin induced apoptosis pathway and it is mitochondria-dependent.

In mammalian cells, apoptosis pathway can be divided into caspases- dependent and -independent pathway and caspases plays an important role in the initiation of apoptosis [36]. Caspases involving apoptosis can be grouped into "apoptotic initiator," such as caspase-8, and "apoptotic effector," such as caspase-3 based on the substrate specificities and target proteins of caspases [30]. In this study, curcumin induced apoptosis was observed and via ER stress which led to mitochondria dysfunction due to the curcumin increasing the levels of Bax (pro-apoptotic protein) and decreasing the levels of Bcl-2 which is a anti-apoptotic protein, and those effects are followed by the activation of caspase-3. Caspase-3 activity was analyzed by flow cytometric analysis (Fig. 5). Curcumin induced DNA damage in examined A-549 cells (Fig. 4) was based on the Comet assay and this damage caused increased levels of P53 and p21 caused the effects of Cdc2 and cyclin B and curcumin promoted the Wee1 CHK2 and cdc25c then led to G2/M arrest. Our results from Western blot also showed that curcumin induced the increase of p53 levels in A-549 cells. It is known that p53 protein can activate the transcription of a variety of apoptosis-associated genes, and to program cell death in response to genotoxic stresses [34, 35]. Therefore, understanding of the cell cycle and its relationship to p53 are critical for the successful clinical development of these agents for the treatment of patients with lung cancers.

These actions (curcumin promoted caspase-3 activation) showed a time-subsequent and a dose-corresponding manner. We also showed that the added

caspase-3 inhibitor, Ac-DEVD-CHO, and inhibited curcumin-induced caspase-3 activity led to decrease the apoptosis in these examined cells (Fig. 5). In conclusion, these results demonstrated that curcumin-induced G2/M arrest through DNA damage led to increase the levels of P53 and p21 which affect CDC2 and cyclin B and promoted the levels of Wee1 and CHK2 which affect Cdc25c before leading to G2/M arrest (Fig. 13). Curcumin induced apoptosis in A-549 cells through the mitochondria dysfunction leading to the release of cytochrome c and involves a caspase-3-mediated mechanism (Fig. 13).

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FIGURE LEGENDS

Figure 1. The morphological changes and percentages of the viable A-549 cells after treated with curcumin. The A-549 cells ($2x10^5$ cells/well; 12-well plates) were plated in 90% Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate + 10% FBS with 30 μM curcumin for 6, 12, 24 and 48 h. The cells were photographed (panel A) by pharse-contrast microscope and collected by centrifugation and the viable cells were determined by trypan blue exclusion and flow cytometry (panel B) as described in Materials and Methods. Each point is mean ± S.D. of three experiments. *P<0.05

Figure 2. The cell cycle arrest and sub-G1 group of A-549 cells after treated with curcumin. The A-549 cells were exposed to 0, 5, 10, 20 and 30 μM curcumin for 48 h and the cells were harvested and analyzed for cell cycle (panel A: the percent of cells in phase). The A-549 cells were treated with 30 μM curcumin for various time periods then were analyzed for the percent of cells in different cell pharse and apoptosis (panel B) as described in Materials and Methods. Data represents mean ± S.D. of three experiments. *P<0.05

Figure 3: Apoptosis of A-549 cells treated with curcumin were examined by using DAPI staining. The A-549 cells were incubated with 30 μM curcumin for 6, 12, 24 and 48 h and apoptosis were determined by using DAPI staining then photographed by fluorescence microscope as described in Materials and Methods

Figure 4. Curcumin induced DNA damage in human lung carcinoma A-549 cells was examined by Comet assay. The A-549 cells ($5x10^3$ cells/well; 12- well plates) were

incubated with various concentrations of curcumin for 24 hours and DNA damage was determinated by Comet assay as described in Materials and Methods.

Figure 5. Effects of caspase-3 inhibitor (Ac-DEVD-CHO) on curcumin induced caspase-3 activity, MMP and apoptosis in human lung cancer A-549 cells. The A-549 cells were treated with various concentrations of curcumin or were pre-treated with caspase-3 inhibitor (Ac-DEVD-CHO) for 3 hours then treated with 30 μM curcumin before cells were harvested for caspase-3 activity and the levels of MMP and apoptosis determinations as described in Materials and Methods (Panel A: caspase-3 activity; panel B: levels of MMP; panel C: apoptosis). Data represents mean ± S.D. of three experiments. *P<0.05.

Figure 6. Flow cytometric analysis of reactive oxygen species (ROS) in A-549 cells with curcumin for various time periods. The A-549 cells ($5x10^5$ cells/ml) were treated with 30 μM curcumin for 0, 15, 30, 45, 60, 90 and 120 minutes to detect the changes of ROS. The zero concentration was defined as control. The percentage of cells that were stained by DCFH-DA dye, and the stained cells were determined by flow cytometry as described in the Materials and Methods section. *differs between curcumin and control. *P<0.05

Figure 7. Flow cytometric analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in A-549 cells with curcumin. The A-549 cells ($5x10^5$ cells/ml) were treated with 0, 5, 10, 20, 30 and 50 μM curcumin for 24 hours before cells were harvested for $\Delta\Psi_m$ assay. The zero concentration was defined as control. The percentage of cells that were stained by DiOL6 dye, and the stained cells were determined by flow cytometry

as described in the Materials and Methods section. *differs between curcumin and control. *P<0.05

Figure 8. Flow cytometric analysis of Ca^{2+} concentration in A-549 cells with with 30 μM curcumin for various time periods. The A-549 cells ($5x10^5$ cells/ml) were treated with 30 μM curcumin for 0, 6, 12, 24 and 48 h to detect the changes of Ca^{2+} concentration. The zero concentration was defined as control. The percentage of cells that were stained by Indo-1/AM dye, and the stained cells were determined by flow cytometry as described in the Materials and Methods section. *differs between curcumin and control. *P<0.05

Figure 9. Effects of calcium antagonist BAPTA (Ca^{2+} chelator) on curcumin affect the levels of Ca^{2+}, MMP and apoptosis in A-549 cells. Flow cytometric analysis on the effects of BAPTA on the levels of Ca^{2+}, $\Delta\Psi_m$ and apoptosis in A-549 cells after treated with curcumin. The A-549 cells were pre-treated with BAPTA for 3 h then treated with 30 μM curcumin before cells were harvested for Ca^{2+} (Panel A), $\Delta\Psi_m$ (Panel B) and apoptosis (Panel C) determination as described in Materials and Methods. Data represents mean ± S.D. of three experiments. *P<0.05.

Figure 10. Representative Western blot showing changes on the levels of Wee1, CHK1, Cdc25c, p53, p21, Cdc2, Cyclin B1,cspase-3 and -9, Bcl-2, COX-1 and -2, NF-kB, catalase, SOD(Zn and Cu), MMP-2 and -9 in A-549 cells after treated with curcumin. The A-549 cells ($5x10^6$/ml) were treated with 30 μM curcumin for 24 h before cytosolic fraction and total protein were prepared and determined as described in Materials and Methods. Then followed by the evaluation of the levels of associated protein levels (Panel A: Wee1, CHK1, Cdc25c, p53, p21, Cdc2, Cyclin B1;

panel B: caspase-3 and-9; panel C: Bcl-2, COX-1 and -2, NF-kB; panel D: catalase, SOD(Zn and Cu); panel E: MMP-2 and -9) and expressions were estimated by Western blotting as described in Materials and Methods.

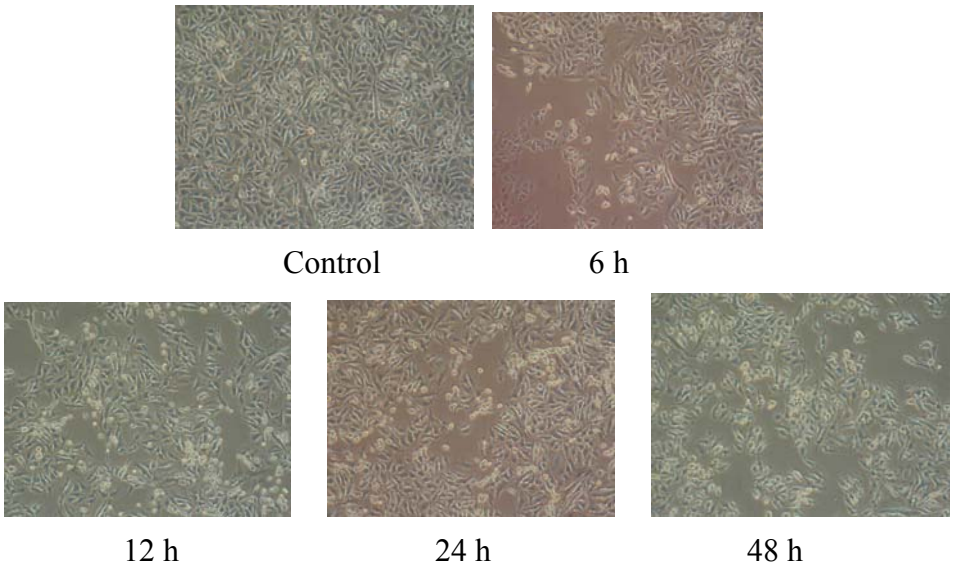
Figure 11. Effects of curcumin on the levels of Gadd153 mRNA in A-549 cells. The A-549 cells ($5x10^5$ cells/ml) were treated with 30 μM curcumin for 0, 1, 3, 6 and 12 h to extract total RNA then PCR were performed as showed in Materials and Methods. The levels of mRNA Gadd153 (Panel A) and ratio of mRNA/GAPDH (panel B) Data represents mean ± S.D. of three experiments. *P<0.05

Figure 12. Effects of curcumin on the cells migration of A-549. The A-549 cells ($2x10^4$ cells/ml) were treated with or without 30 μM curcumin for 12 h then were photographed as showed in Materials and Methods.

Figure 13. Proposed model of curcumin mechanism of action for G2/M arrest and apoptosis in A-549 cells. Curcumin induce p53, p21 expressions before leading to decrease Cdc2, cyclin B1 and promoted Wee1 and CHK2 leading to G2/M arrest. Curcumin increased the production of ROS led to ER stress and Ca^{2+} production and decreased $\Delta\Psi_m$ levels that led to caspase-3 activity before causing apoptosis in A-549 cells.

Fig. 1

A.



B.

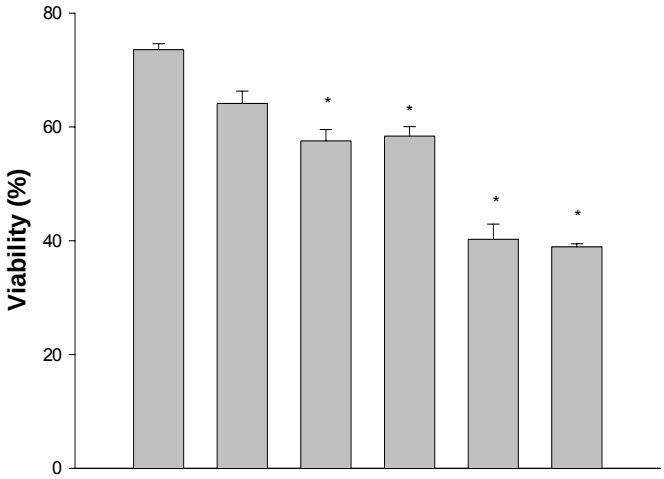
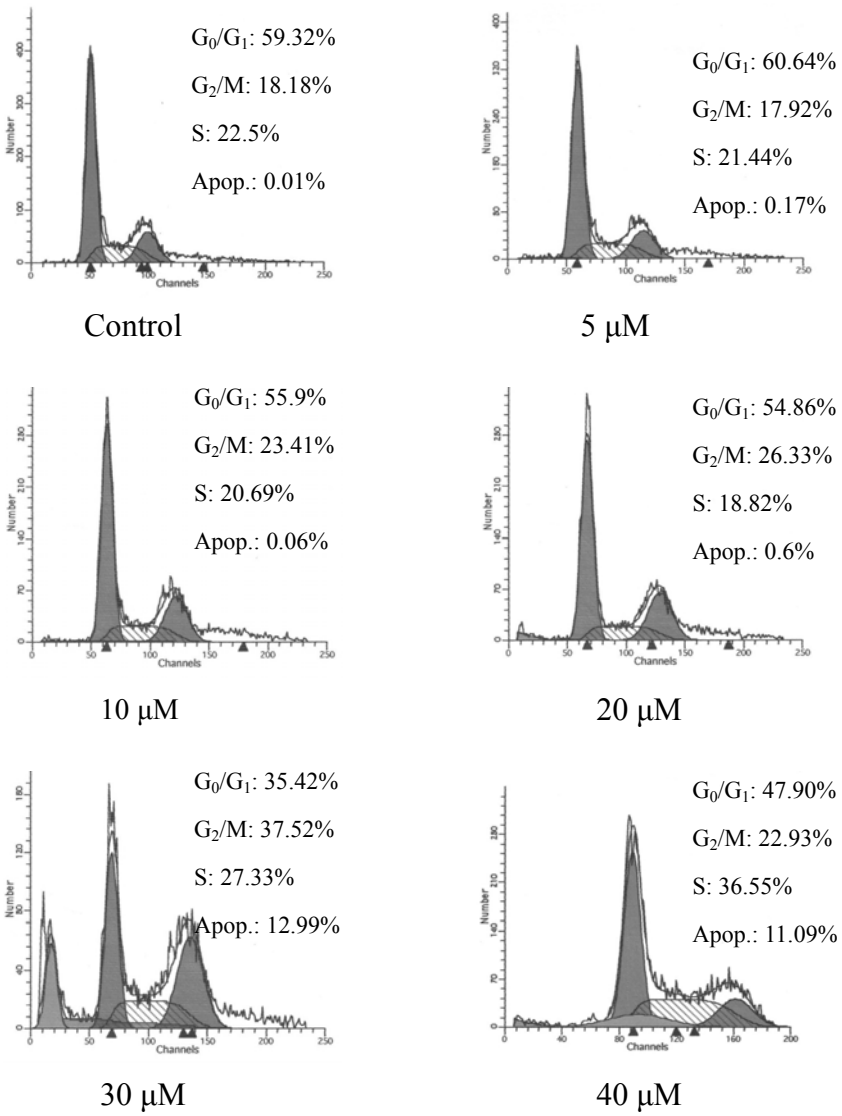


Fig.2

A.


G0/G1: 59.32%
G2/M: 18.18%
S: 22.5%
Apop.: 0.01%
Control
G0/G1: 60.64%
G2/M: 17.92%
S: 21.44%
Apop.: 0.17%
5 μM
G0/G1: 55.9%
G2/M: 23.41%
S: 20.69%
Apop.: 0.06%
10 μM
G0/G1: 54.86%
G2/M: 26.33%
S: 18.82%
Apop.: 0.6%
20 μM
G0/G1: 35.42%
G2/M: 37.52%
S: 27.33%
Apop.: 12.99%
30 μM
G0/G1: 47.90%
G2/M: 22.93%
S: 36.55%
Apop.: 11.09%
40 μM
Number
Channels

B.

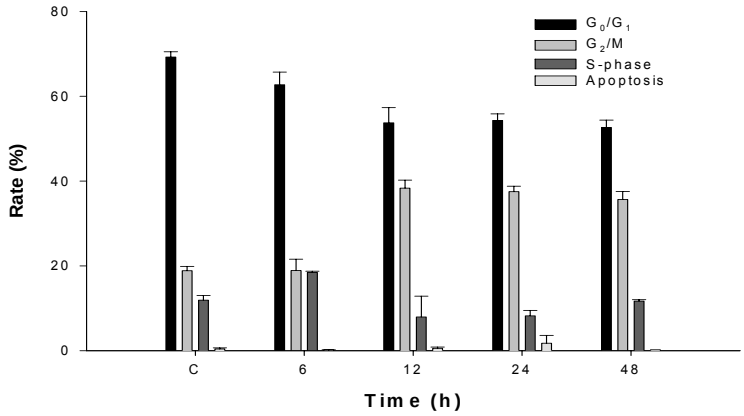

G0/G1
G2/M
S-phase
Apoptosis
Rate (%)
0
20
40
60
80
C
6
12
24
48
Time (h)

Fig. 3

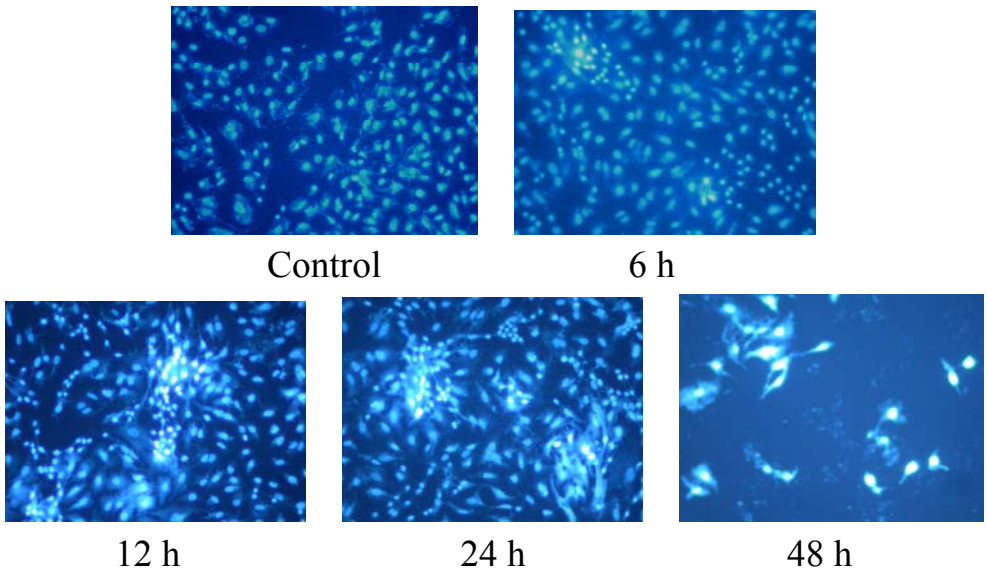


Fig. 4

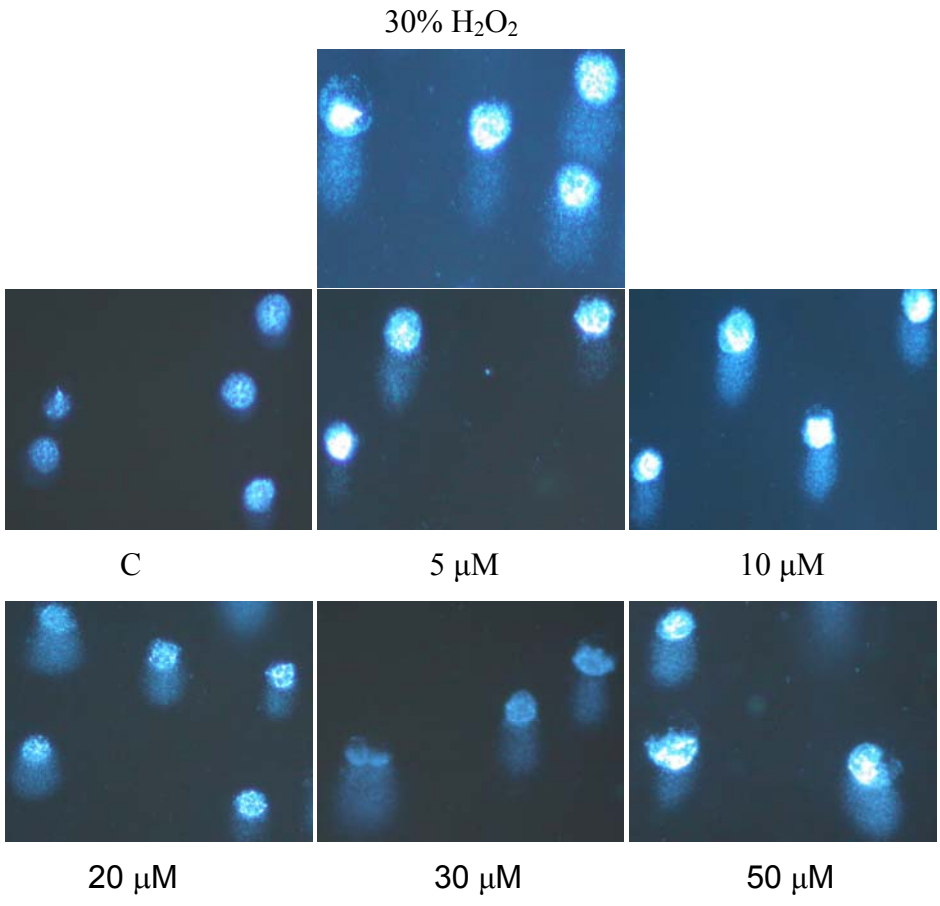
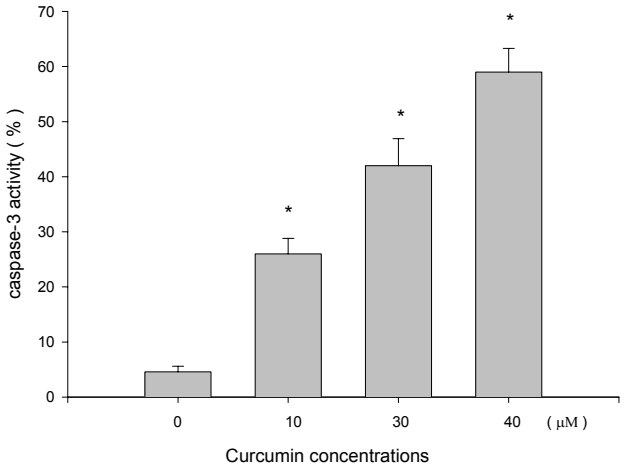
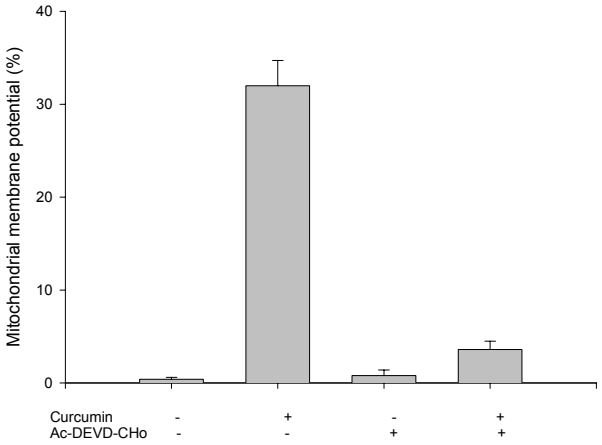


Fig. 5

A


caspase-3 activity (%)
70
60
50
40
30
20
10
0
*
*
*
0
10
30
40
(μM)
Curcumin concentrations

B


Mitochondrial membrane potential (%)
40
30
20
10
0
Curcumin - + - +
Ac-DEVD-CHo - - + +

C

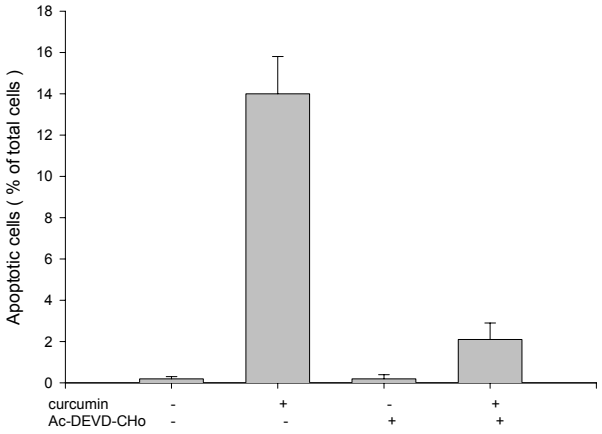

Apoptotic cells (% of total cells)
18
16
14
12
10
8
6
4
2
0
curcumin - + - +
Ac-DEVD-CHo - - + +

Fig. 6

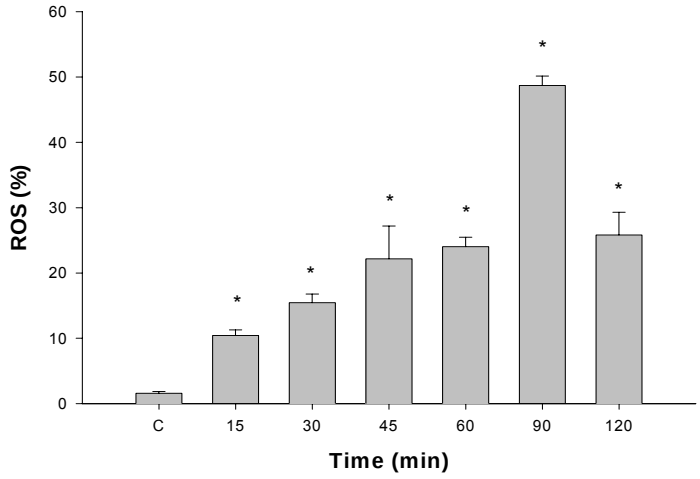


Fig. 7

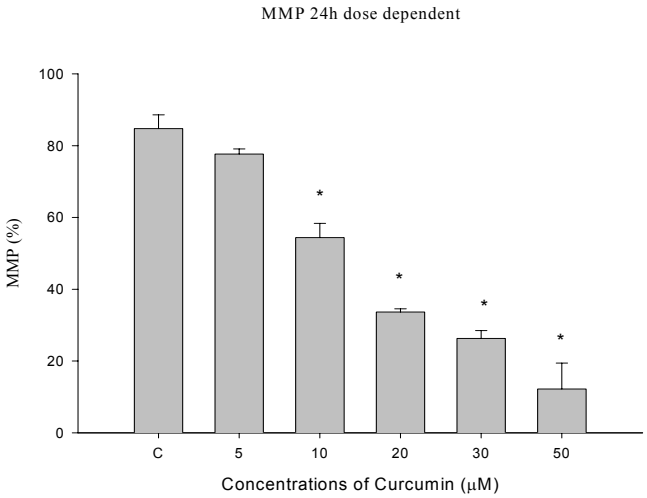


Fig. 8

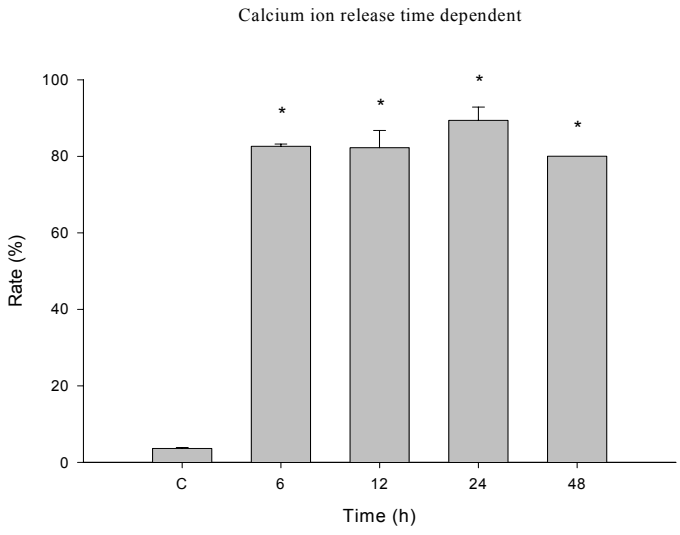
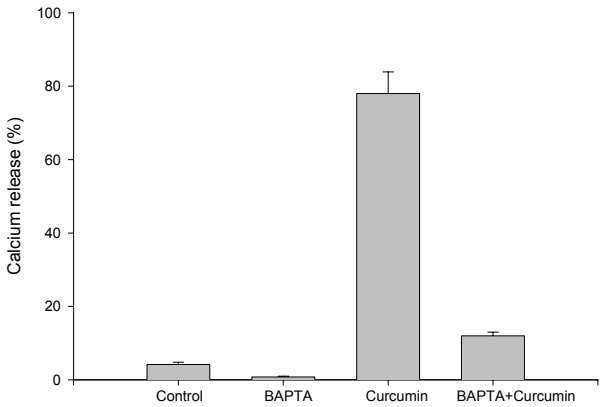
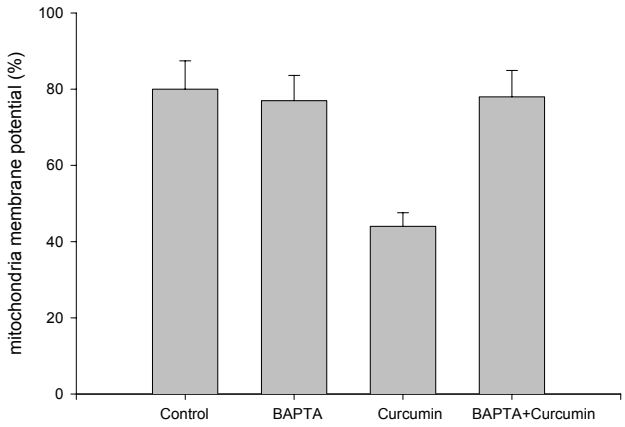


Fig. 9

A


Calcium release (%)
100
80
60
40
20
0
Control
BAPTA
Curcumin
BAPTA+Curcumin

B


mitochondria membrane potential (%)
100
80
60
40
20
0
Control
BAPTA
Curcumin
BAPTA+Curcumin

C

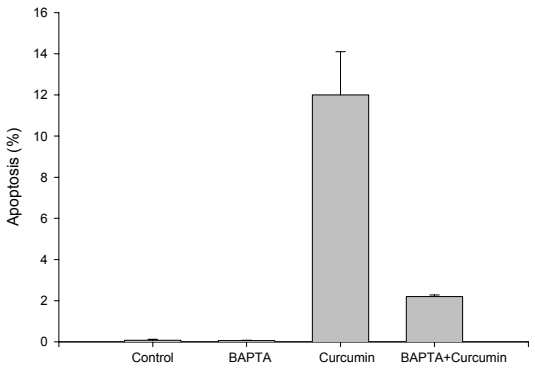
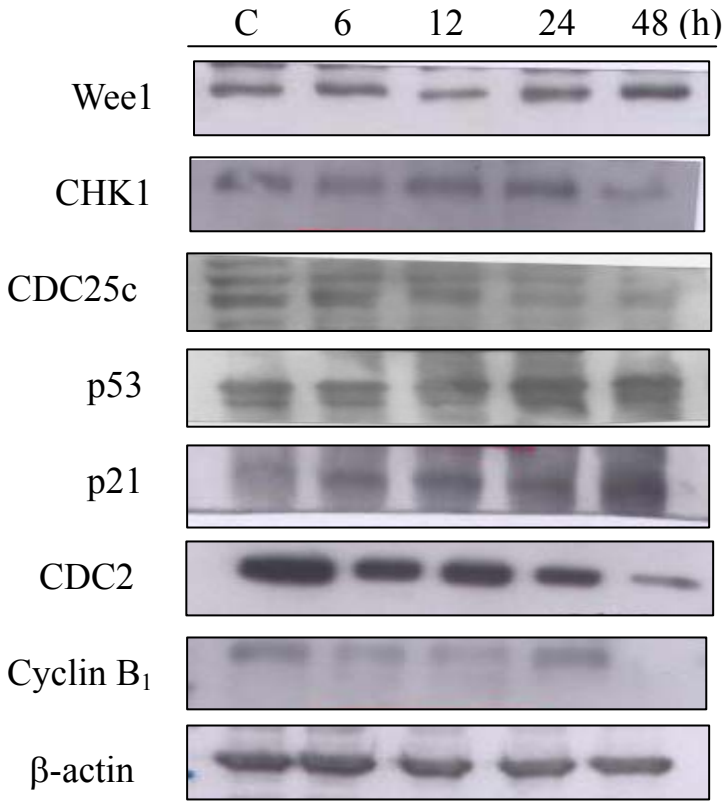
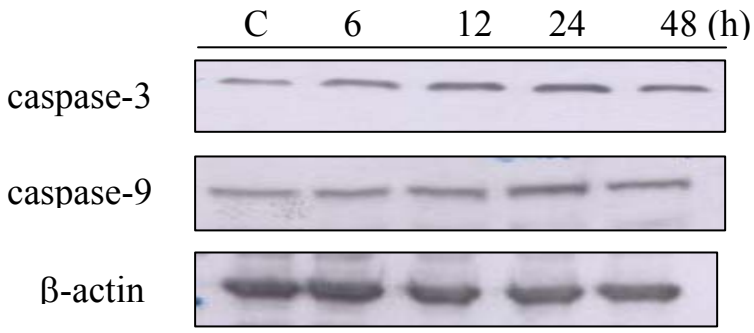

Apoptosis (%)
16
14
12
10
8
6
4
2
0
Control
BAPTA
Curcumin
BAPTA+Curcumin

Fig. 10

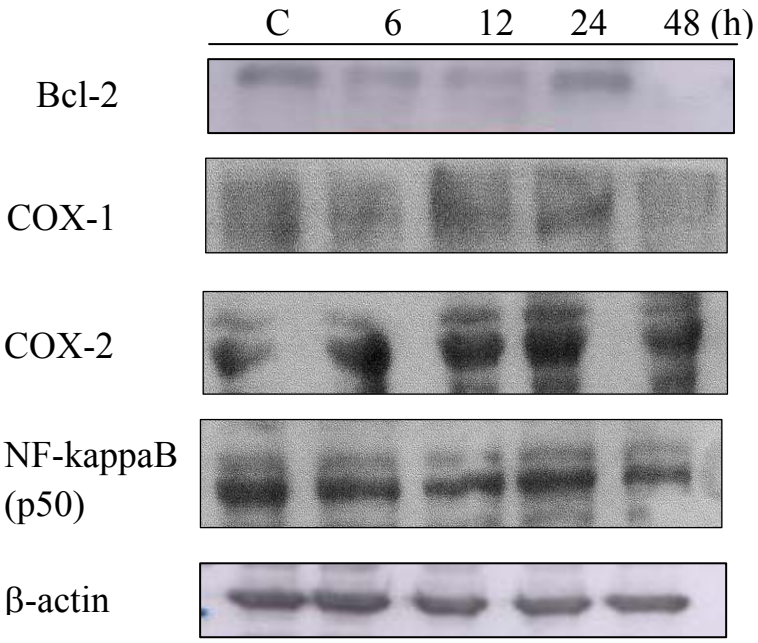
A.


C
6
12
24
48 (h)
Wee1
CHK1
CDC25c
p53
p21
CDC2
Cyclin B1
β-actin

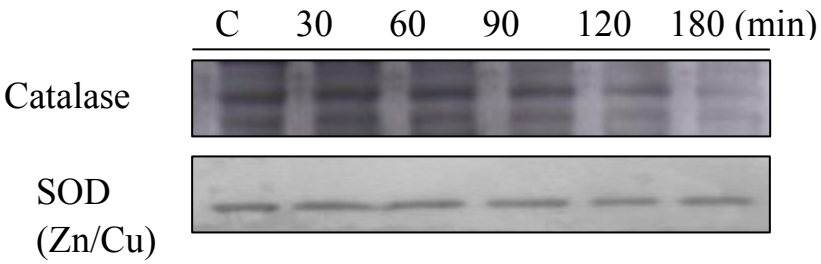
B.


C
6
12
24
48 (h)
caspase-3
caspase-9
β-actin

C.


C
6
12
24
48 (h)
Bcl-2
COX-1
COX-2
NF-kappaB
(p50)
β-actin

D.


C
30
60
90
120
180 (min)
Catalase
SOD
(Zn/Cu)

E.

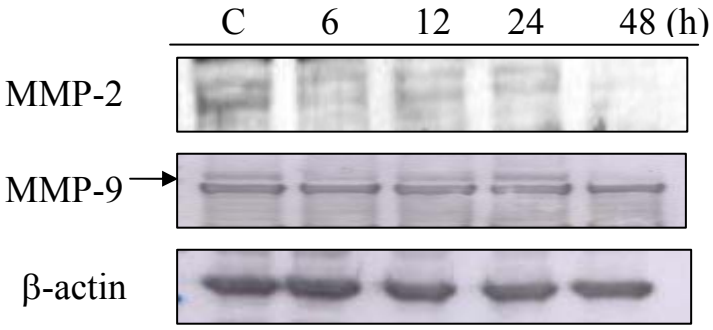
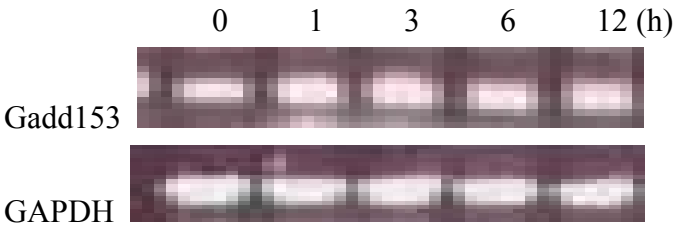

C
6
12
24
48 (h)
MMP-2
MMP-9
β-actin

Fig. 11

A


0
1
3
6
12 (h)
Gadd153
GAPDH

B

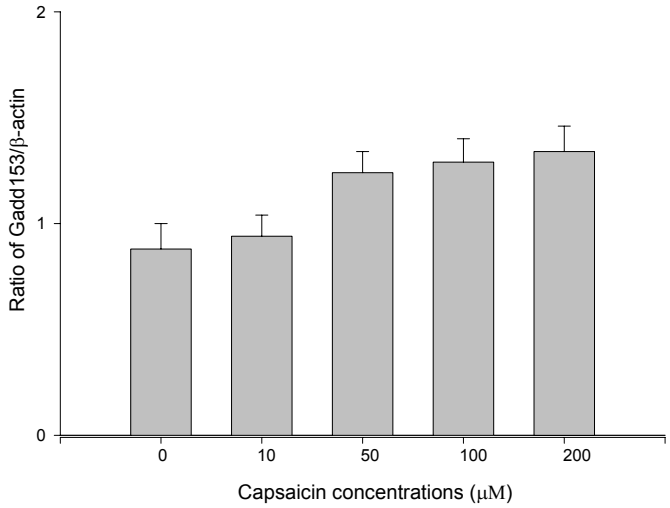

Ratio of Gadd153/β-actin
2
1
0
0
10
50
100
200
Capsaicin concentrations (μM)

Fig. 12

Cell migration assay

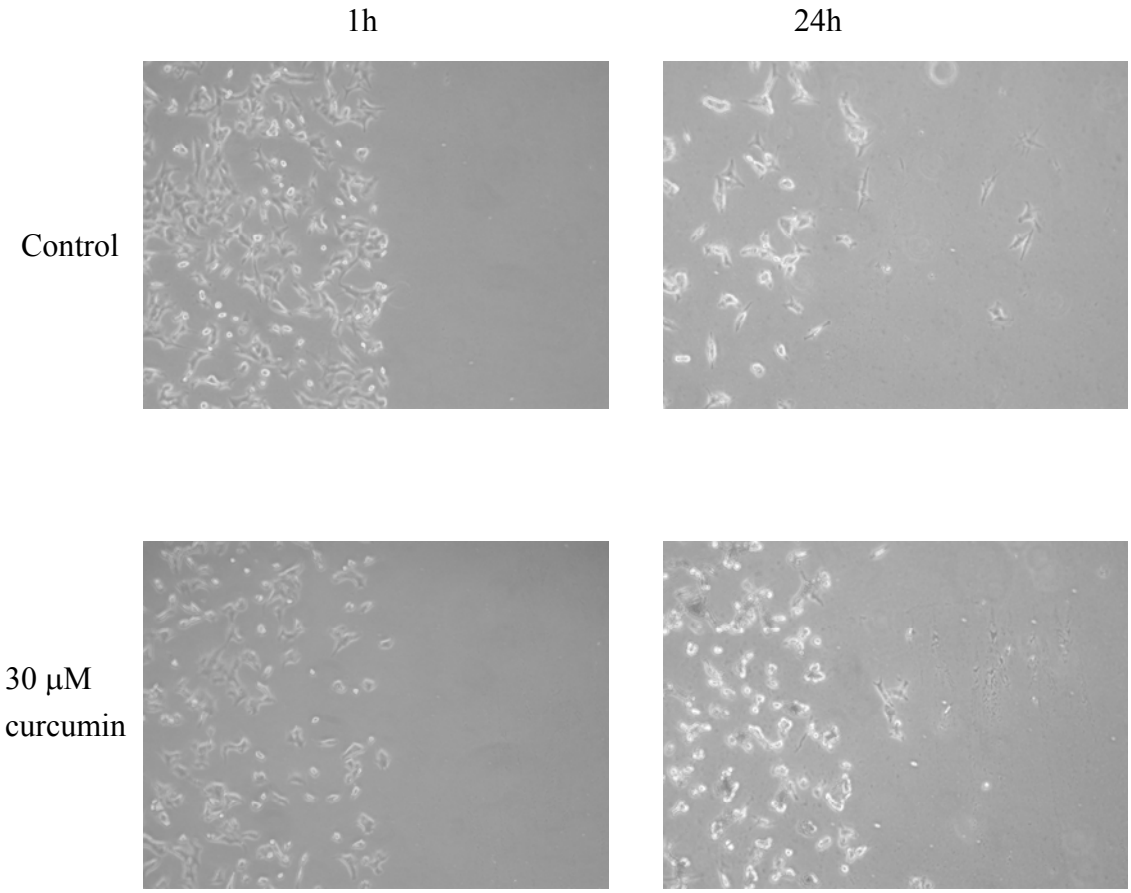
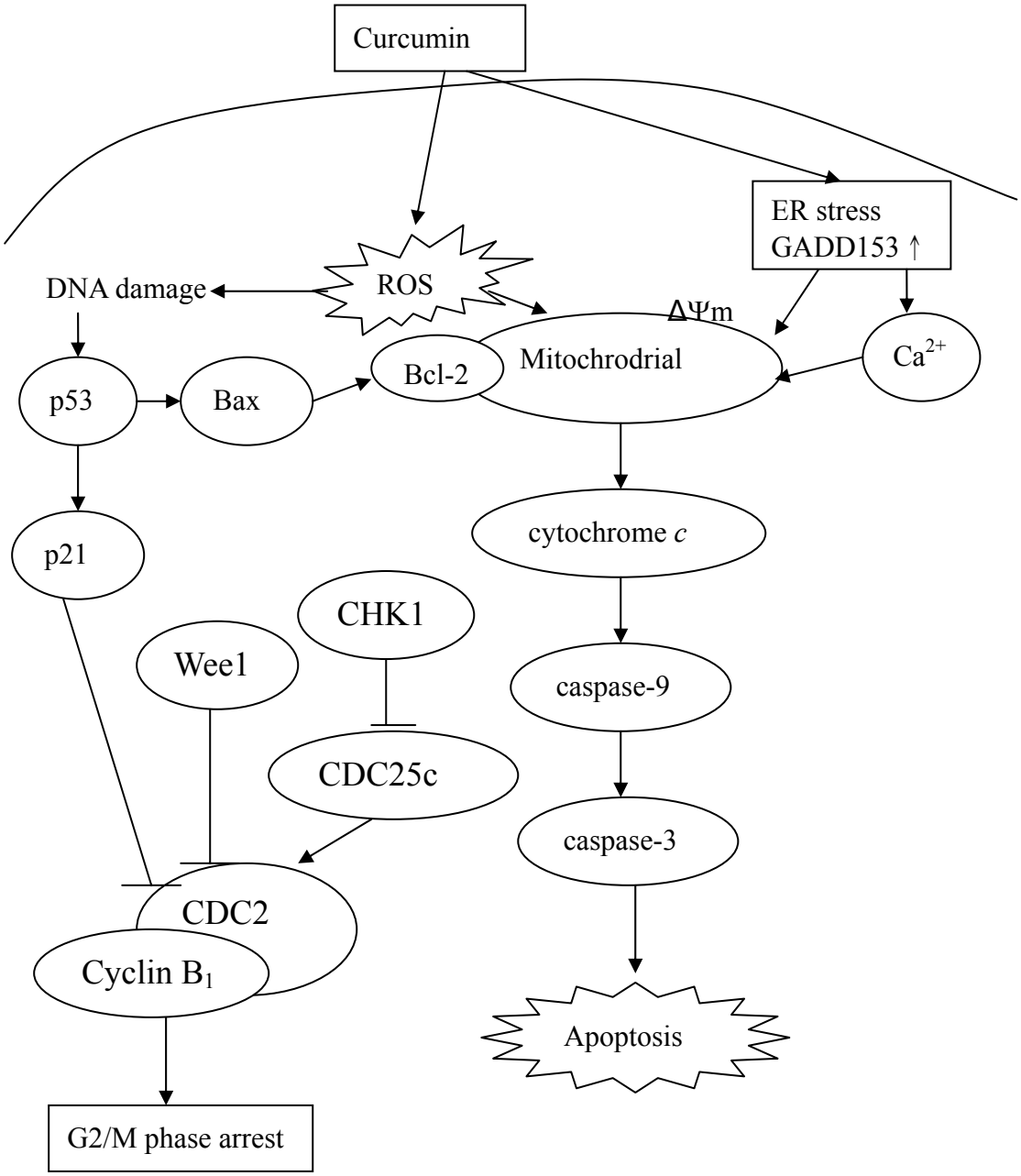


Fig. 13


Curcumin
ER stress
GADD153 ↑
ROS
DNA damage
ΔΨm
Bcl-2
Mitochrodrial
Ca^{2+}
p53
Bax
p21
cytochrome *c*
CHK1
Wee1
caspase-9
CDC25c
caspase-3
CDC2
Cyclin B_1
Apoptosis
G2/M phase arrest