

Induction of Apoptosis by Caffeine Is Mediated by the p53, Bax, and Caspase 3 Pathways¹

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ABSTRACT

Caffeine is a key component of many popular drinks, especially tea and coffee. Previous reports have shown that caffeine may contribute to the chemopreventive effect of tea in animals. Here, we report that treatment with low concentrations of caffeine induced apoptosis in JB6 Cl41 cells. JB6 Cl41 cells were starved in 0.1% fetal bovine serum/MEM for 72 h and then treated with 50–450 μ M caffeine for 24 h. Cells showed the typical DNA laddering pattern and other characteristics of apoptosis. The IC₅₀ of caffeine on JB6 Cl41 cells was 2.7 mM. Induction of apoptosis by caffeine appeared to be p53-dependent because cells lacking p53 (*p53*^{-/-}) showed no signs of apoptosis after treatment with caffeine. Immunoprecipitation assays and Western blot analysis showed that caffeine induced phosphorylation of p53 at Ser¹⁵ in JB6 Cl41 cells. The same low concentration of caffeine that was effective for inducing phosphorylation of p53 was also shown to increase p53 activation. Expression of Bax, another p53 target, distinctly increased in a time- and dose-dependent manner. Cleaved caspase 3 was also increased in a time- and dose-dependent manner. These data show that a low concentration of caffeine can induce p53-dependent apoptosis in JB6 cells through the Bax and caspase 3 pathways.

INTRODUCTION

Caffeine (methylxanthine), a purine alkaloid, is a key component of many popular drinks, most notably tea and coffee. Caffeine was shown to substantially increase apoptosis and mitochondrial damage in PD184352-treated, a mitogen-activated protein kinase kinase inhibitor, human cancer cell lines HL-60 and U937 (1). Caffeine has also been shown to inhibit ATM³ and ATR kinase activities (2, 3) and contribute to prevention of cancer (4). Caffeine has certain physiological properties and is well known as an antisleep agent because of its excitatory effects on nervous-humoral regulation. Caffeine also has been shown to increase radiosensitivity in mouse embryo cells (5) and the HeLa cell line (6), inhibit UVB-induced skin carcinogenesis in mice (4), and increase the sensitivity of cells to certain chemotherapeutic agents (7). Moreover, reports indicate that high concentrations of caffeine directly induce cellular apoptosis (8–10), but its use clinically has not been possible because of the undesirable side effects associated with high concentrations. The mechanisms of caffeine's anticarcinogenesis effects are not well understood but could be related to its effects on cell proliferation, apoptosis, angiogenesis, and/or the immune system (4).

The tumor suppressor gene, *p53*, functions as a key component of a cellular emergency response system to induce cell growth arrest or apoptosis (11, 12). Activation of p53 can result either in a G₁ cell cycle arrest or apoptosis that contributes to suppression of malignant transformation. The maintenance of genomic integrity and inactivation

of p53 in cancers may be responsible for accelerated cell growth and resistance to genotoxic anticancer agents (12, 13). Dubrez *et al.* (14) reported that caffeine sensitizes the human non-small cell lung cancer H358 cell line to p53-mediated apoptosis by inducing mitochondrial translocation and a conformational change in the BAX protein. Lu *et al.* (15) also reported that topical application of caffeine to SKH-1 mice immediately after exposure to UVB increased the apoptotic response but only had a very small effect on increases in expression of wild-type *p53*. Administration of caffeine p.o. or topically had no effect on the levels of p53 or apoptosis in control or non-UVB-treated mice. Bache *et al.* (16) reported that caffeine completely prevents the irradiation-induced G₂-M arrest in the human sarcoma cell lines, US8-93 and LMS6-93, which both have a p53 mutation. However, another study also showed that in bladder cancer cell lines, caffeine-increased radiosensitivity is not dependent on G₂-M arrest or apoptosis (17). These conflicting results suggest that the relationship between caffeine and p53 still remains unclear.

The *Bax* gene is an apoptosis-promoting member of the *bcl-2* gene family. The Bcl-2 protein is known to form heterodimers with the Bax protein *in vivo* and the molar ratio of Bcl-2 to Bax determines whether apoptosis is induced or inhibited in several tissues (18). The Bax protein controls cell death through its participation in disruption of mitochondria and subsequent cytochrome *c* release and is also considered to be one of the primary p53 targets (19). However, Lowe *et al.* (20) reported that overexpression of Bax protein by a prostate-specific promoter can induce apoptosis in human prostate carcinoma LNCaP cells and indicated that *Bax* gene therapy is a promising approach for the treatment of prostate cancer.

Caspase 3 is well known as one of the key executioners of apoptosis. Activation of caspase 3 requires proteolytic processing of its inactive zymogen into activated p17 and p19 subunits (21). Bax drives the release of cytochrome *c* from the mitochondria and cytochrome *c* release activates caspase 3 (22). Cleaved caspase 3 is regarded as a primary mechanism of apoptosis. However, the function of caspase 3 in the pathway of caffeine-induced apoptosis is not clear.

Although previous studies showed that caffeine induces cellular apoptosis, the cells were treated with very high concentrations (up to 5 mM) of caffeine (8–10, 23). In this study, we determined whether low concentrations of caffeine can induce apoptosis and explored the mechanism for that induction. Our data show that caffeine induced apoptosis in a p53-dependent manner in JB6 Cl41 cells, a well-developed cell culture model for studying tumor promotion. In addition, we found that p53 activation and phosphorylation of p53 at Ser¹⁵ in JB6 Cl41 cells were also increased by exposure to low concentrations of caffeine. Moreover, the typical characteristics of apoptosis were observed in *p53*^{+/+} cells but not *p53*^{-/-} cells. The expression level of Bax and cleaved caspase 3 also increased in JB6 Cl41 cells treated with low concentrations of caffeine.

MATERIALS AND METHODS

Plasmids and Reagents. The *p53* luciferase reporter plasmid (PG13-Luc) was constructed as described previously (24). Eagle's MEM, DMEM, FBS, L-glutamine, and gentamicin were from Life Technologies, Inc. (Grand Island, NY). Luciferase assay substrate was purchased from Promega (Madison, WI).

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³ The abbreviations used are: ATM, ataxia telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3-related protein; FBS, fetal bovine serum; PI, propidium iodide.

Caffeine and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of caffeine in water were stored at -20°C . The mouse monoclonal IgG against p53 (Ab-1) antibody was from Oncogene Co. (La Jolla, CA); phospho-specific p53 (Ser¹⁵) antibody was from New England Biolabs, Inc. (Beverly, MA). Bax antibody was from Santa Cruz Co. (Santa Cruz, CA), and cleaved caspase 3 antibody was from Cell Signal Technology, Inc. (Beverly, MA). Enhanced Chemifluorescein Western blotting kits were from Amersham Pharmacia Biotech (Piscataway, NJ). Modified-Lowry protein assay reagent was from Sigma (St. Louis, MO). Folin & Ciocalteus Phenol Reagent was from Pierce (Rockford, IL) and polyvinylidene difluoride membrane from Millipore (Bedford, MA).

Cell Culture and Induction of Apoptosis. The JB6 mouse epidermal cell line C141 and its stable p53-luciferase reporter plasmid transfectant, C141 p53 cells, were cultured in monolayers with MEM supplemented with 5% (v/v) heat-inactivated FBS and glutamine (2 mM) at 37°C in a humidified atmosphere of 5% CO_2 . p53^{+/+} and p53^{-/-} fibroblast cells were cultured in DMEM supplemented with 10% FBS and glutamine (2 mM) at 37°C in a humidified atmosphere of 5% CO_2 . For Western blot analysis, JB6 C141 cells, p53^{+/+} and p53^{-/-} cells were starved in 0.1% FBS/MEM and 0.5% FBS/DMEM respectively, and then treated with caffeine. For analysis of apoptosis, all cells were starved for 72 h and then treated with caffeine for another 24 h.

Measurement of Apoptotic Cells by Flow Cytometry. Cytotoxic action of caffeine was determined using the Annexin V-FITC Apoptosis Detection Kit (Medical & Biological Laboratories, Nagoya, Japan) according to the instruction protocol provided. Briefly, cells were trypsinized, washed once with serum-containing MEM, and incubated with Annexin V-conjugated FITC and PI in binding buffer at room temperature for 5 min in the dark. Stained cells were analyzed by a flow cytometer (FACSCalibur; Becton-Dickinson).

DNA Fragmentation Analysis. After cells were starved for 72 h and then treated with different concentrations of caffeine for another 24 h, both detached and attached cells were harvested by scraping and centrifugation (1500 rpm 10 min at 4°C). The cells were disrupted with lysis buffer [5 mM Tris (pH 8.0), 0.5% Triton X-100, and 20 mM EDTA] on ice for 30 min and then centrifuged at 14,000 rpm at 4°C for 45 min. The supernatant fractions were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) three times and once with chloroform and precipitated with absolute ethanol plus 5 M NaCl at -20°C for at least 1 h. The precipitants were centrifuged at 14,000 rpm at 4°C for 30 min, washed twice with 70% ethanol, redissolved in Tris/EDTA buffer containing 100 $\mu\text{g}/\text{ml}$ RNase, and incubated at 37°C for 1 h. The DNA fragments were separated by 1.8% agarose gel electrophoresis and visualized under UV light.

Immunofluorescence Staining Analysis. After caffeine treatment, cells were fixed in 0.5% paraformaldehyde for 5 min at room temperature, washed twice in PBS, and stained with Hoechst 33342 (5 $\mu\text{g}/\text{ml}$) for 1 h under shielded light conditions. Morphological changes were observed using fluorescence microscopy, and the percentage of apoptotic cells was scored counting at least 200 cells. The same experiment was repeated three times, and the average percentage of apoptotic cells was determined for each concentration of caffeine.

Cell Proliferation Assay. Cell proliferation was analyzed by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) following the instruction manual provided. Briefly, 5000 JB6 C141 cells were seeded in 96-well plates. After 24 culturing in 5% FBS/MEM at 37°C in a 5% CO_2 incubator, cells were starved in 0.1% FBS/MEM for 72 h under the same culture conditions. Cells were treated or not treated with caffeine for 24 h and then MTS was added, and the cells were incubated for 2 h at 37°C in a humidified, 5% CO_2 atmosphere. Absorbance of a colored formazan product proceeded by dehydrogenase enzymes in metabolically active cells was recorded at 492 and 690 nm as a background. Data were calculated as a percentage of control cells that were not treated with caffeine. The concentration of caffeine corresponding to 50% cell death indicates IC_{50} .

Assay for p53 Activity. In brief, 8×10^3 viable C141 p53 luciferase reporter cells were suspended in 100 μl of 5% FBS/MEM and added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 . After culturing 24 h, cells were starved in 0.1% FBS/MEM for 72 h. The cells were then exposed to different concentrations of caffeine for induction of p53 activity. The cells were extracted with lysis buffer, and luciferase activity was measured using a luminometer (Monolight 2010). The results are expressed as relative p53 activity.

Immunoprecipitation and Immunoblotting. Immunoblotting was carried out as described previously (24). In brief, JB6 C141 cells were cultured to 80% confluence and then starved in 0.1% FBS/MEM for 72 h at 37°C . The media were changed to fresh 0.1% FBS/MEM, and the cells were incubated for another 2 h at 37°C . Cells were then treated or not treated with different concentrations of caffeine for various time periods and then disrupted with lysis buffer [20 mM Tris-HCl, (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM β -glycerolphosphate, 1 mg/ml leupeptin, 1 mM Na_2VO_4 , 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] and centrifuged at 14,000 rpm for 10 min in a microcentrifuge. The lysates containing 400 μg of protein were immunoprecipitated using a monoclonal mouse IgG antibody against p53 and rotated overnight at 10 cycles/min at 4°C . Then, protein A/G plus-agarose beads were added and continuously rotated for another 3 h at 4°C . The beads were washed three times with PBS. The levels of phosphorylated p53 protein (Ser¹⁵), total p53, Bax, and cleaved caspase 3 proteins were selectively measured by Western blot analysis using specific antibodies. Antibody-bound proteins were detected by chemiluminescence and analyzed using the Storm 840 Scanner (Molecular Dynamics, Sunnyvale, CA).

Western Blotting. To determine protein expression level, cells were washed once with cold PBS and disrupted with lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 mM NaF, 1 mM Na_3VO_4 , and 0.5 mM phenylmethylsulphonyl fluoride] for 30 min on ice. Protein content was determined by the Modified-Lowry Protein Assay method, and 50 μg of each cell lysate were used for PAGE, and proteins were transferred onto a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked by incubating the membranes in blocking buffer (5% nonfat milk in PBS, 0.1% Tween 20) at room temperature for 1 h. Membranes were then incubated with the primary antibody diluted in buffer (1 \times PBS containing 5% BSA, 0.1% Tween 20, 0.05% NaN_3) overnight at 4°C . The membranes were then washed with 0.25% Tween 20 in PBS at room temperature for 15 min and then washed with only PBS for 5 min. Membranes were incubated with the secondary antibody, which is an alkaline phosphatase-linked rabbit IgG. Protein expression level was analyzed by the Storm 840 scanner. To verify equal protein loading and transfer, membranes were reprobed for β -actin using an antiactin rabbit polyclonal antibody.

RESULTS

Apoptosis Is Induced in JB6 C141 Cells by Low Concentrations of Caffeine. Previous studies indicated that a high concentration of caffeine induced apoptosis (7–9, 23). Qi *et al.* (25) reported that 5 mM caffeine induced apoptosis in a human lung cancer cell line and that it could synergistically enhance radiation-induced apoptosis. Meikrantz *et al.* (23) also reported that apoptosis was induced in HeLa cells by 5 mM caffeine. In contrast, our data indicate that apoptosis was induced by caffeine at relatively low concentrations in JB6 C141 cells. After JB6 C141 cells were starved in 0.1% FBS/MEM 72 h, cells underwent apoptosis visualized as typical DNA laddering (Fig. 1A) and changes in morphology (Fig. 1, B–F) when treated with caffeine (16.5–450 μM). The percentage of apoptotic cells was gradually increased as caffeine concentration increased (Fig. 1G). Using annexin V-conjugated FITC and PI stain to analyze the percentage of apoptotic cells induced by caffeine, we found that the late apoptotic cells (UR) or early apoptotic cells (LR), respectively, increased from 6.8 to 15.8% or 6.3 to 8.1% when cells were treated for 24 h with caffeine from 50 to 450 μM . Total percentage of apoptotic cells (UL + LR) increased from 13.1 to 23.9% (Fig. 2 B–D). However, in untreated control JB6 C141 cells, UR and LR, respectively, reached 6.3 and 4.7% (Fig. 2A). However, the maximum concentration of caffeine used in JB6 C141 cells was 450 μM , a concentration that was far below IC_{50} of 2.7 mM (Fig. 3).

Low Concentrations of Caffeine Induce Phosphorylation of p53 at Ser¹⁵ in Vivo. Previous studies indicated that JB6 C141 cells contain the wild-type p53 protein (26, 27). To determine whether p53 is phosphorylated at Ser¹⁵ in JB6 C141 cells treated with caffeine, we used a phospho-specific antibody against p53 at Ser¹⁵ to do immu-

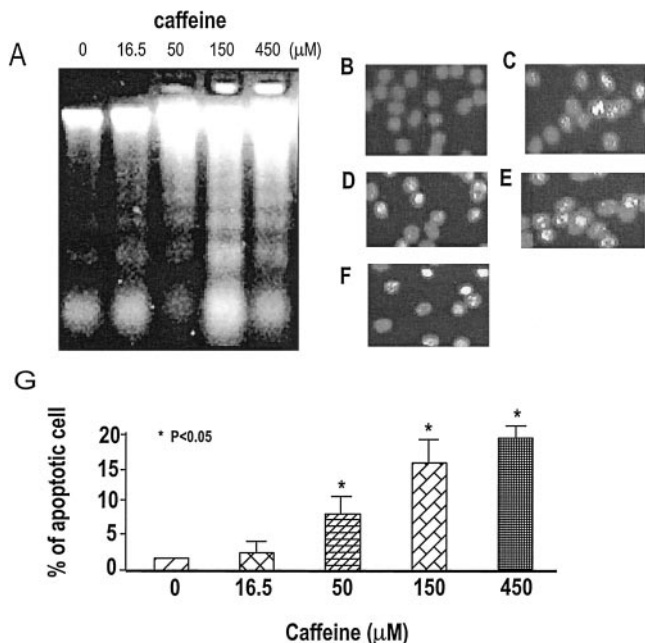


Fig. 1. Low concentrations of caffeine induce apoptosis in JB6 C141 cells. Fragmented DNA was separated by 1.8% agarose gel electrophoresis and visualized under UV light (A). Morphological changes in apoptotic cells were observed under fluorescence microscopy, and the percentage of apoptotic cells was scored counting at least 200 cells (B–F). The experiments were repeated at least three times, and the average percentage of apoptotic cells for each concentration of caffeine is shown (G).

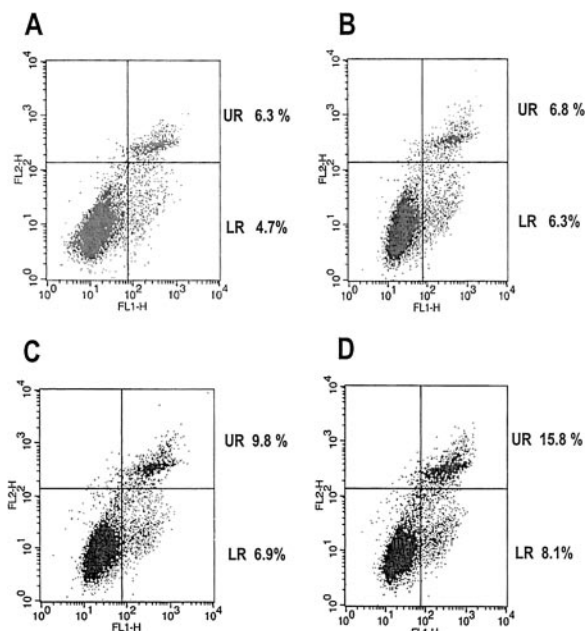


Fig. 2. Caffeine increased the percentage of apoptotic cells in JB6 C141 cells. The Annexin V-FITC Apoptosis Detection kit was used to analyze apoptotic cells. Cells were incubated with Annexin V-conjugated FITC and PI in binding buffer at room temperature for 5 min. Stained cells were analyzed by flow cytometry (FACS caliber; Beckman). A indicates the control cells without caffeine treatment. The percentage of apoptotic cells increased in JB6 C141 cells when cells were treated with caffeine 50 μM (B), 150 μM (C), or 450 μM (D) for 24 h. UR indicates the percentage of late apoptotic cells (PI-stained cells), and LR indicates the percentage of early apoptotic cells (annexin V-stained positive cells).

noblot analysis. Our data showed that the level of p53 phosphorylation at Ser¹⁵ was increased by 1.5-fold within 1 h after cells were treated with caffeine and reached a maximal induction of 5-fold after 4–6 h (Fig. 4A). Immunoblotting showed that the increased level of p53

protein (Fig. 4B) corresponded well with the increased level of p53 phosphorylation at Ser¹⁵. These results indicated that caffeine induced p53 phosphorylation at Ser¹⁵.

Low Concentrations of Caffeine Increase p53-dependent Transcriptional Activation. Many studies indicate that normal function of p53 is crucial for the induction of apoptosis in human and murine cells (28, 29). Irradiation-induced apoptosis of thymocytes was almost completely blocked in p53-deficient mice (30). To investigate the possible role of p53 in caffeine-induced apoptosis, we used C141 p53 luciferase reporter cells to explore transcriptional activation after cells were treated with caffeine. We found that transcriptional activation gradually increased in JB6 C141 p53 luciferase promoter cells treated with caffeine at doses ranging from 16.5 to 450 μM (Fig. 5).

Caffeine Induces Apoptosis in p53^{+/+} Cells but not in p53^{-/-} Cells. To obtain direct evidence that p53 is required for induction of apoptosis by caffeine, we tested the effects of caffeine on two fibroblast cell lines derived from mouse embryos containing either wild-type p53 (p53^{+/+}) or that were p53 deficient (p53^{-/-}; Ref. 31). The results of DNA fragmentation analysis showed that the typical DNA laddering pattern (Fig. 6A) and morphological changes occurred in p53^{+/+} cells (Fig. 6, B–F) after treatment with caffeine. No sign of apoptosis was observed in p53^{-/-} cells even when treated with caffeine at 450 μM (Fig. 6, G–K). On the other hand, the percentage

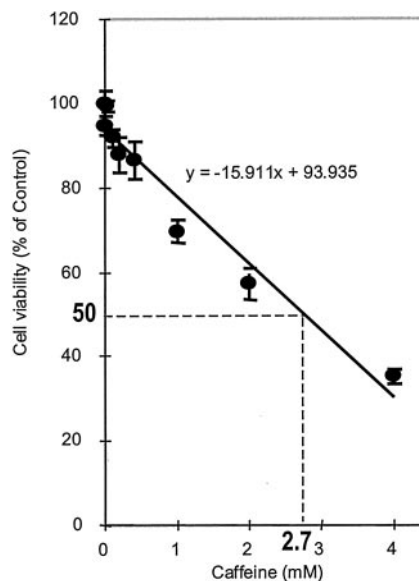


Fig. 3. Caffeine inhibits JB6 C141 cellular proliferation. Cells were treated as previously described in “Materials and Methods.” Absorbance of a colored formazan product was recorded at 492 and 690 nm as a background. Data were calculated as a percentage of control in which cells were untreated. The concentration of caffeine corresponding with 50% cell death indicates IC₅₀.

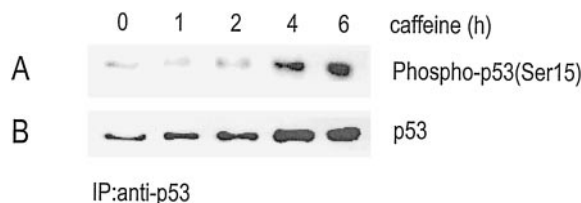


Fig. 4. A low concentration of caffeine induces phosphorylation of p53 at Ser¹⁵ *in vivo*. Serum-starved C141 cells were treated with 450 μM caffeine for the indicated periods of time. Cell lysates were prepared from these cells, and 400 μg of protein were used for immunoprecipitation of p53 and detection of phosphorylation of p53 at Ser¹⁵ using a specific antibody (A). Blots were stripped and reprobed using an antibody against total p53 (B).

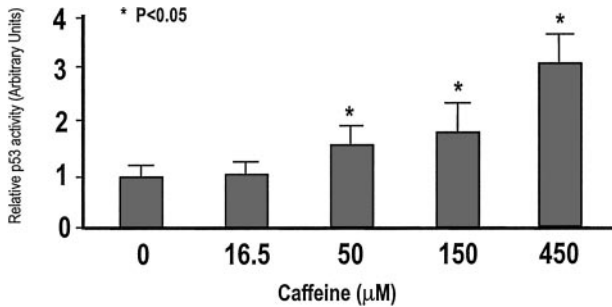


Fig. 5. Low concentrations of caffeine increase p53 activity. Viable C141-p53 luciferase reporter cells (8×10^3) were suspended in $100 \mu\text{l}$ of 5% FBS/MEM and then added to each well of a 96-well plate. After culturing for 24 h, cells were starved in 0.1% FBS/MEM for an additional 72 h. Cells were then exposed to different concentrations of caffeine for 24 h. Cells were disrupted with lysis buffer and luciferase activity was measured using a luminometer (Monolight 2010). Data were calculated as a percentage of control cells that were untreated. The average activity was determined from three separate experiments. * indicates a significant increase in p53 activity versus control ($P < 0.05$).

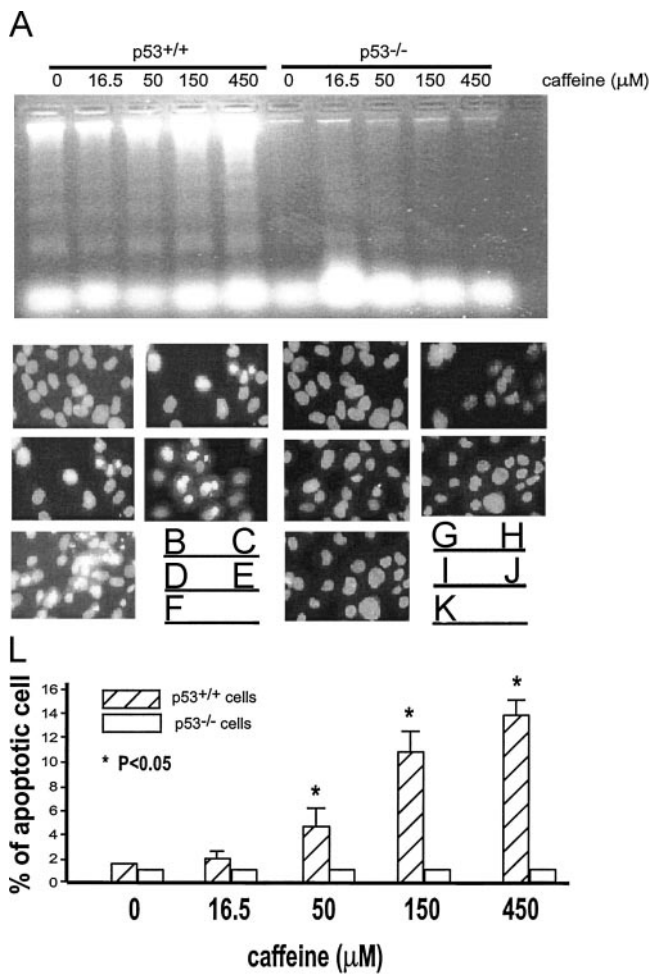


Fig. 6. Caffeine induces apoptosis in wild-type p53 ($p53^{+/+}$) but not in p53-deficient ($p53^{-/-}$) cells. Both $p53^{+/+}$ and $p53^{-/-}$ fibroblast cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in 10% FBS/DMEM supplemented with glutamine (2 mM). All cells were starved in 0.5% FBS/DMEM for 72 h and then treated with caffeine for another 24 h. DNA fragmentation (A) and the morphological changes (B-K) were determined using the same methods as described for Fig. 1. The percentage of apoptotic cells was calculated as the average of three experiments (L).

of apoptotic cells gradually increased in $p53^{+/+}$ cells treated with increased concentrations of caffeine (Fig. 6L) but not in $p53^{-/-}$ cells.

Caffeine Induces Bax Expression in JB6 C141 Cells. Studies indicate that Bax accelerates programmed cell death and correlates

with p53 status (18, 32). In this study, the Bax protein levels were determined by Western blot using a specific antibody against Bax. Compared with the control, Bax expression increased in JB6 C141 cells treated with caffeine in a time and dose-dependent manner (Fig. 7, A and C). Beta actin expression levels indicated equal sample loading amount (Fig. 7, B and D).

Caffeine Induces Bax Expression in $p53^{+/+}$ Cells but not in $p53^{-/-}$ Cells. To determine whether caffeine acts upstream or downstream of Bax in a p53-dependent apoptotic pathway, $p53^{+/+}$ and $p53^{-/-}$ fibroblast cells were treated with caffeine, and Bax expression was analyzed by Western blot. Our results showed that treatment with increasing concentrations of caffeine also significantly increased the protein level of Bax in $p53^{+/+}$ cells but not in $p53^{-/-}$ cells (Fig. 8). These data indicate that loss of p53 almost completely blocks the expression of Bax stimulated by caffeine and that p53 plays an important role in caffeine-induced apoptosis.

Caffeine Induces Cleaved Caspase 3 Expression in JB6 C141 Cells. To explore whether caffeine induces apoptosis by activation of caspase, we used a specific antibody to test for cleavage of caspase 3, which is a marker of caspase-dependent apoptosis. Our results show that the cleaved caspase 3 expression level is increased in a time- and dose-dependent manner (Fig. 9). Compared with control cells, cleaved caspase 3 expression increased in a time- (Fig. 9A) and dose-dependent manner (Fig. 9C), but the β -actin expression levels were not changed (Fig. 9, B and D). These results indicate that activated caspase 3 is another mediator in the induction of apoptosis by caffeine.

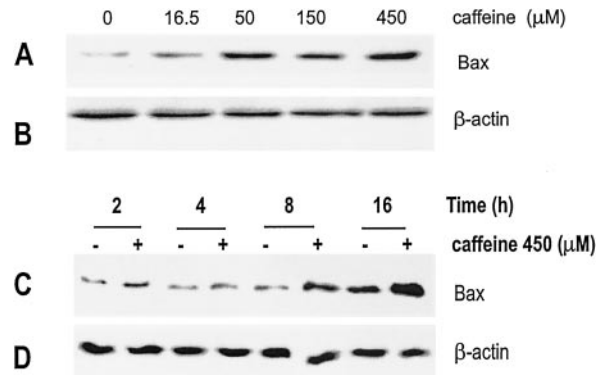


Fig. 7. Caffeine induces an increased expression of Bax protein in JB6 C141 cells. JB6 C141 cells were starved in 0.1% FBS/MEM for 72 h and then treated with different concentrations of caffeine for the indicated times. Changes in Bax protein expression were determined by Western blot analysis. Caffeine induced Bax expression in a dose- (A) and time-dependent (C) manner in JB6 C141 cells. β -Actin was used as an internal control to monitor equal protein sample loading (B and D).

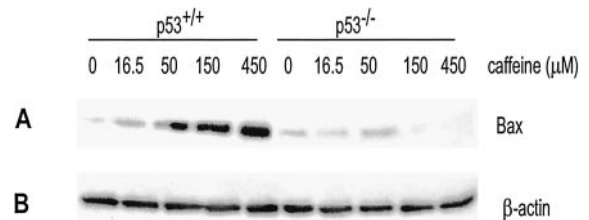


Fig. 8. Caffeine induces an increased expression of Bax protein in $p53^{+/+}$ but not in $p53^{-/-}$ cells. $p53^{+/+}$ and $p53^{-/-}$ cells were starved in 0.5% FBS/DMEM for 72 h and then treated with different concentrations of caffeine for another 16 h. Lysates were prepared from these cells, and protein amount was determined using the Modified-Lowry Protein Assay kit. Bax protein levels were determined by Western blot analysis using the corresponding antibody (A). β -Actin was used as an internal control to monitor equal protein sample loading (B).

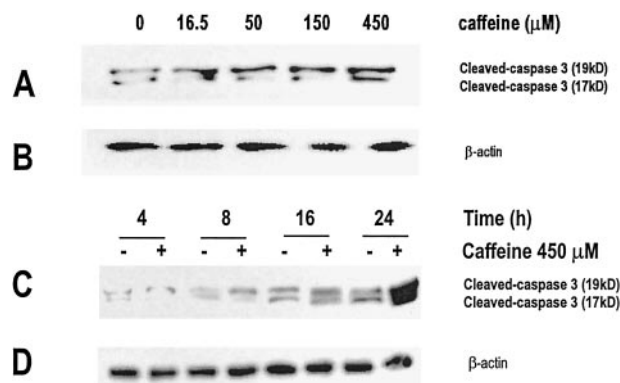


Fig. 9. Caffeine induces an increased expression of cleaved caspase 3 in dose- and time-dependent manners in JB6 C141 cells. JB6 C141 cells were starved in 0.1% FBS/MEM for 72 h and then treated with different concentrations of caffeine for the indicated times. Cleaved caspase 3 protein levels were determined by Western blot analysis using a cleaved caspase 3 antibody (A and C). β -Actin was used as an internal control to monitor equal protein sample loading (B and D).

DISCUSSION

Chemoprevention is now regarded as a promising strategy to control cancer (33), and many natural or dietary substances have been shown to inhibit experimental carcinogenesis (34). Caffeine is one such dietary chemopreventive phytochemical that has recently attracted great interest because of its remarkable multifunctional inhibitory effects on carcinogenesis in cultured cells or animal models (3, 4, 8, 9, 14, 17, 25). Jang *et al.* (35) reported that 10 mM caffeine induced apoptosis in the human neuroblastoma cell line SK-N-MC. However, this high concentration of caffeine may not be relevant to physiological conditions for the chemoprevention of cancer. In this study, apoptotic cells were confirmed by DNA ladder analysis, morphological changes, and flow cytometry (Figs. 1 and 2). The maximum concentration for induction of apoptosis was 450 μ M, which was much lower than the IC_{50} of 2.7 mM (Fig. 3). A recent study indicated that low concentrations of caffeine (<5 mM) prevented apoptosis of macrophages from rat lung, whereas moderate concentrations of caffeine (5–20 mM) induced apoptosis in these cells, and caffeine induced apoptosis in these cells in a dose- and time-dependent manner (36). In our study, low concentrations (450 μ M) of caffeine-induced apoptosis in JB6 C141 cells after cells were starved in 0.1% FBS/MEM for 72 h. These data demonstrated that caffeine-induced apoptosis may most likely be dependent on cell type or type of extracellular stimuli (36, 37).

To explore the possible mechanisms of caffeine in induction of apoptosis, we determined p53 phosphorylation and activity in JB6 C141 cells, which contain wild-type p53 (26, 27, 31). Our data showed that phosphorylation of p53 at Ser¹⁵ increased in JB6 C141 cells after cells were treated with increasing concentrations of caffeine (Fig. 4). p53 activity also increased in these cells (Fig. 5). Phosphorylation of p53 at Ser¹⁵ is regarded as a critical step for p53-dependent transactivation (38). Our previous report showed that the phosphorylation of p53 at Ser¹⁵ could be induced by phenethyl isothiocyanate (26) or by resveratrol (31, 36, 39), which is regarded as a promising agent for cancer prevention. To additionally confirm that p53 is involved in caffeine-induced apoptosis, we compared caffeine's effect in wild-type p53 fibroblast cells ($p53^{+/+}$) and p53-deficient fibroblast cells ($p53^{-/-}$). Our data showed that caffeine only induced apoptosis in $p53^{+/+}$ cells and not in $p53^{-/-}$ cells (Fig. 6). These results indicate that in JB6 C141 cells, apoptosis induced by caffeine is p53-dependent and caffeine-induced p53 phosphorylation at Ser¹⁵ may be attributed to stabilization of p53.

Several pathways mediate p53-induced apoptosis, and one of these involves the Bax protein, which is a p53 target and a proapoptotic member of the Bcl-2 family of proteins (40–42). We show here that Bax accumulated in JB6 C141 cells in a dose- and time-dependent manner after cells were treated with caffeine (Fig. 7). The above results suggest that p53 phosphorylation or activity may be related to the accumulation of Bax. So, we additionally investigated the effect of caffeine on Bax protein expression in $p53^{-/-}$ and $p53^{+/+}$ fibroblast cells. Our data show that the level of Bax protein increased only in $p53^{+/+}$ fibroblast cells but not in $p53^{-/-}$ fibroblast cells after caffeine treatment (Fig. 8). This result additionally confirms that p53 is upstream of Bax and that Bax is a p53 downstream effector (43). Moreover, these results also suggest that p53 is not only a functional regulator of Bax but also can cause overexpression of Bax in JB6 C141 and $p53^{+/+}$ fibroblast cells.

Bax can promote the cytosolic release of cytochrome *c*, which in turn, activates caspase 3, one of the key executioners of apoptosis (44). In this study, we found that cleaved caspase 3 protein expression increased when JB6 C141 cells had been starved for 72 h and then treated with caffeine for another 24 h (Fig. 9). Therefore, caspase 3 activation is also involved in the process of apoptosis induced by caffeine. Activated caspase 3 cleaves its substrate and marks the beginning of DNA cleavage (21). However, many questions remain to be answered, including how caffeine enters the cells and the identity of its target on the cell surface or inside the cell.

In conclusion, these data illustrate that the mechanism of induction of apoptosis in JB6 C141 cells by caffeine involves activated p53, Bax, and caspase 3. Low dose of caffeine can also induce apoptosis in JB6 C141 cells under certain conditions, and thus, caffeine, a content of tea, may be a powerful agent to prevent cancer.

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