Downregulation of survivin and activation of caspase-3 through the PI3K/Akt pathway in ursolic acid-induced HepG2 cell apoptosis

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Ursolic acid (UA), a naturally occurring pentacyclic triterpene, is a potent in-vitro anticancer agent, acting through control of growth, apoptosis and differentiation. As the mechanism of its proapoptotic effects on human hepatocellular carcinoma cells has not been extensively studied, we performed an in depth evaluation of the effects of UA on apoptosis in human HepG2 cells. UA was found to inhibit the proliferation of HepG2 cells in a concentration and time-dependent manner. After treatment, cells showed evidence of activation of apoptosis, including the presence of apoptotic bodies and DNA fragmentation. UA-induced apoptosis was accompanied by a significant decrease in bcl-2 and survivin expression, with the corresponding ratio of bax/bcl-2 increased. The treatment with UA also increased the protein level and enzymatic activity of caspase-3. Z-DEVD-fmk, a specific caspase-3 inhibitor, significantly inhibited both the cytotoxic effect and the DNA fragmentation induced by UA, demonstrating the requirement for caspase-3 activity in UA-induced apoptosis. Inactivation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway was also involved, as inhibition of PI3K by LY294002 significantly increased UA-induced apoptosis. Kinetic experiments indicated that UA downregulated PI3K/p85 subunit (PI3K/p85) and phospho-Akt, before downregulating survivin. The further results also confirmed that LY294002 not only downregulated survivin alone, but considerably enhanced the repression of survivin combined with UA. UA therefore seemed to downregulate the expression of survivin by blocking PI3K/Akt. Taken together, the data suggest that the proapoptotic effect of UA on HepG2 cells is mediated by activation of caspase-3, and is highly correlated with inactivation of PI3K/Akt/survivin pathway. *Anti-Cancer Drugs* 20:249–258 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Hepatocellular carcinoma is the fifth most common human malignancy, affecting approximately one million people annually around the world. It ranks third in the cancer-related deaths, causing more than 600 000 deaths globally each year [1]. The overproliferation of hepatocellular carcinoma cells is partly because of a deficiency in apoptosis; therefore, searching for agents that can induce apoptosis in these cells may lead to potential clinical treatments for this disease [2].

Ursolic acid (UA), a pentacyclic triterpene acid, is widely distributed in dietary vegetables and is well known to possess a wide range of biological functions [3,4]. Earlier studies have shown that UA can inhibit proliferation and induce apoptosis in many kinds of cancer cells, including breast, leukemia, prostate, lung, melanoma, and endometrial cancer cells [5–10]. UA is also able to regulate the cell cycle and the induction differentiation, in addition to inhibiting tumor metastasis and angiogenesis [11,12]. Recently, Slamenová *et al.* [13] found that nontoxic concentrations of UA could reduce the genotoxicity of the effective anti-HIV drug 3′-azido-3′-dideoxythymidine in HepG2 cells. UA can also prevent DNA damage and proliferation of HepG2 cells by modification of apoptosis [14]; however, the mechanism of its proapoptotic effect on HepG2 cells remains poorly understood.

Apoptosis (programmed cell death) is an active physiological process of cellular self-destruction, which is accompanied by specific morphological and biochemical changes in the nucleus and cytoplasm. These changes, which reflect the operation of an intracellular death process that silently eliminates no-longer-needed cells without an inflammatory response, involve a number of genes, including the bcl-2 family, the caspase family, and the IAP (inhibitor of apoptosis protein) family [15]. Bcl-2 is a prooncogene that serves as the link between apoptosis and cancer. Connected to an immunoglobulin locus by a chromosome translocation in follicular

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lymphoma, bcl-2 was found to inhibit cell death, rather than to promote proliferation [16,17]. Apoptosis also involves a cascade of proteolytic reactions affected chiefly by the caspase family, for example, caspase-3, the major executor of apoptosis. IAPs such as survivin enhance survival of tumor cells primarily through suppression of apoptosis-related cell death, perhaps by direct inhibition of caspase-related proteins [18]. The phosphatidylinositol 3-kinase-Akt (PI3K/Akt) pathway also appears to be important in the development and proliferation of various human cancers. The PI3K/Akt pathway is involved in the inhibition of apoptosis by inactivating some proapoptotic proteins, such as BAD and caspase-9, whereas some antiapoptotic proteins are activated by this pathway [19,20]. Whether the PI3K/Akt pathway is involved in regulating UA-induced HepG2 cell apoptosis or not has not yet been determined.

In this study, we investigated the proapoptotic effect of UA on HepG2 cells with the aim of discovering the underlying signaling pathway involved in regulating UA-induced apoptosis. UA inhibited proliferation and induced apoptosis in HepG2 cells in a concentration and time-dependent manner. UA treatment could also increase the activity of caspase-3, caspase-8, caspase-9 and downregulate the expression of bcl-2 and survivin. In light of the role of survivin as an inhibitor of apoptosis in many cancers, we further evaluated the effect of the PI3K/Akt pathway on UA-induced apoptosis and survivin regulation.

Materials and methods

Reagents and cell culture

UA (purity $>98\%$) was prepared as a 10 mmol/l stock solution in sterilized dimethylsulfoxide (DMSO; Sigma, USA) and stored at -20°C. Hoechst 33258 dye and LY294002 were bought from Sigma. Polyclonal antibodies against β-actin, p53, survivin, bax, and bcl-2 were purchased from Boster Biological technology Inc. (Wuhan, China). Polyclonal antibodies against bcl-xL, caspase-3, caspase-8, caspase-9, PI3K/p85, phospho-Akt and Akt were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% (v/v) penicillin–streptomycin (10 000 U/ml; Sigma, USA) and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C, passed three times weekly. For all experiments except methyl thiazolyl tetrazolium (MTT) assay, exponentially growing cells were allowed to adhere at a concentration of 2×10^5 cells/ml and grow for 24 h in culture medium before exposure to UA. The cells of control group (0 μmol/l) in all experiments were treated with DMSO at a concentration of 0.3%. In the experiment of DNA fragmentation assay and flow cytometry analysis, 3 μmol/l of gambogic acid was used as the positive control [21]. At the end of the incubation interval, cells were collected for analysis described below.

Methyl thiazolyl tetrazolium assay

Cell viability was determined by measuring the absorbance of MTT dye staining of living cells, as described earlier [22]. In brief, cells were seeded at a density of 7.5×10^3 cells/well on 96-well plates and then incubated under various conditions as indicated. After each period of incubation, six wells were selected from each group of cells every day for MTT (50 μg/well) assay. After the cells had been incubated at 37°C for 4 h, the reaction was stopped by adding 150 μl/well of DMSO, and the cells were then incubated for another 10 min. The color reaction was quantified using an automatic plate reader (Bio Rad, USA) at 570 nm with a reference filter of 630 nm. Absorbance is directly proportional to the number of viable cells in the cultures. The inhibition ratio (I%) was calculated using the following equation:

$$\mathrm{I}\% = \left(\frac{A_{\mathrm{control}} - A_{\mathrm{treated}}}{A_{\mathrm{control}}}\right) \times 100\%$$

The IC_{50} value was taken as the concentration that caused 50% inhibition of cell proliferation.

Hoechst staining

The nuclear changes were analyzed by fluorescent microscopy using Hoechst staining, according to the manufacturer's instruction. UA at different concentrations was added and incubated for 24 h. Cells were washed twice with PBS and immobilized in cold 100% MetOH for 5 min. Later, the cells were washed with PBS again and incubated in 1 ml of Hoechst 33258 solution (10 ng/ml in PBS) for 10 min in darkness. The samples were washed in PBS and visualized by fluorescent microscopy (DMRB; Leica Microsystems, Germany).

DNA fragmentation analysis

DNA fragmentation was detected after the extraction of genomic DNA from cells incubated under indicated conditions for 24 h by using apoptotic DNA ladder kit (Biotake; Beijing, China). The DNA extracted was separated on 1.2% agarose gel and visualized under ultraviolet illumination after staining with ethidium bromide.

Annexin V-FITC/propidium iodine flow cytometry analysis

Apoptosis was determined by flow cytometry using a commercially available annexin V-FITC/propidium iodine apoptosis detection kit (KeyGen Biotech; Nanjing, China). Briefly, cells were exposed to UA, LY294002, or a combination of UA and LY294002 at indicated concentrations for 24 h. Later, the cells were harvested,

washed twice in cold PBS, and resuspended in 500 µl of binding buffer at 1×10^5 cells/ml. A suspension of 100 µl was taken and incubated with 5 µl of annexin V-FITC and 5 µl of propidium iodine (20 µg/ml) in the dark for 30 min at room temperature. Finally, 400 µl of PBS was added to each sample and analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, New Jersey, USA).

RNA extraction and semiquantitative reverse-transcription PCR

Total RNA was extracted from cells cultured in 10% fetal bovine serum medium without or with 30 µmol/l UA for 12, 24, and 48 h by using Trizol reagent (Gibco-BRL, USA). For each sample, 2 µg of total RNA was reverse transcribed using oligo dT18 primers and M-MLV (Promega, USA) following standard protocols. As a template for PCR, 1 µl of the newly synthesized cDNA was used, which was performed with 1.5 mmol/l $MgCl_2$, 2.5 U Taq polymerase (Promega, USA), and with 20 pmol/l of sense and antisense primers. The primer sequences were p53, 5′-AGCGATGGTCTGGCCCCTCC-3′, and 5′-GCGCCGGTCTCTCCCAGGA-3′; survivin, 5′-CGAC GTTGCCCCCTGCCTG-3′, and 5′-AAGGAAAGCGCAA CCGGACGA-3′; bax, 5′-TCAGGATGCGTCCACCAAGA A-3′, and 5′-TCCCGGAGGAAGTCCAATGTC-3′; bcl-2, 5′-TGTGTGTGGAGAGCGTCAACC-3′, and 5′-TTCAG AGACAGCCAGGAGAAATC-3′; bcl-xL, 5′-TGGACAAT GGACTGGTTGAGC-3′, and 5′-TGGGAGGGTAGAGT GGATGGT-3′; glyceraldehyde-3-phosphate dehydrogenase internal control, 5′-AAGGTCGGAGTCAACGGATT-3′, and 5′-CTGGAAGATGGTGATGGGATT-3′. Amplification procedure was 94°C for 5 min, followed by 26 cycles at 94°C for 30 s, 58°C for 45 s, 72°C for 45 s, finally by 72°C for 10 min. Aliquots of PCR products were electrophoresed on 1.2% agarose gels, and PCR fragments were visualized by ethidium bromide staining. The DNA bands were analyzed with Quantity One software (Bio Rad).

Western blot

After various treatments for indicated intervals, cells were lysed in lysis buffer (50 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 1% Triton X-100, 1% deoxycholic phenylmethylsulfonyl fluoride, 1 µg/ml of aprotinin, and 1 mmol/l of DTT). 20 µg aliquots of the lysates were separated on a 12% SDS-polyacrylamide gel and transferred electrophoretically (Bio Rad) onto a nitrocellulose membrane (Millipore, USA). Blots were blocked for 2 h in blocking buffer and incubated with primary anti-human rabbit polyclonal antibodies (1 : 200) overnight at 4°C, and with anti-human β-actin rabbit polyclonal antibody (1 : 200) as a control. After washing with TBST, appropriate HRP-conjugated secondary antibody (1 : 2000) was added and incubated at 37°C for 1 h and developed with enhanced chemiluminescence detection system (Amersham Pharmacia, UK). Protein levels were quantified by density analysis using Quantity One software (Bio Rad). Relative protein expression levels were deduced from the ratio of the mean values of each band to that of β-actin.

Assay of caspase activity

The activity of caspases was analyzed fluorometrically with corresponding fluorescent substrates according to the manufacturer's instructions (Peptide Institute; Osaka, Japan). Briefly, HepG2 cells were treated with indicated concentrations of UA for 48 h. Harvested cells (1×10^6) were washed once and resuspended in ice-cold PBS and transferred onto a 96-well plate. The cells were lysed by transferring the plate to a −20°C freezer for at least 30 min, then exposed to 50 µmol/l substrate buffer [substrates: Asp-Glu-Val-Asp (DEVD)-7-amino-4-methylcoumarin (AMC) for caspase-3, Ile-Glu-Thr-Asp (IETD)-AMC for caspase-8, and Leu-Glu-His-Asp LEHD)-AMC for caspase-9, respectively]. The enzymatic reaction was carried out at 37°C for 1 h and the fluorescence of the profluorescent substrate cleaved by activated caspase was detected by using a spectrofluorometer (Hitachi, Japan) (excitation 380 nm, emission 460 nm).

Inhibition of caspase-3 by Z-DEVD-fmk

After treatment with UA, Z-DEVD-fmk (Calbiochem; San Diego, California, USA) [23], or a combination of UA and Z-DEVD-fmk at indicated concentrations for 24 h, MTT assay and DNA fragmentation analysis were then performed following the above methods.

Statistical analysis

All statistical analyses were performed by using SPSS10.0 for Windows software package (Chicago, Illinois, USA). Data represent the means ± SD of three independent experiments. Statistical comparisons of the results were made by using analysis of variance. Significant differences ($P < 0.05$) between the means of control and UA-treated group were analyzed by Dunnett's test.

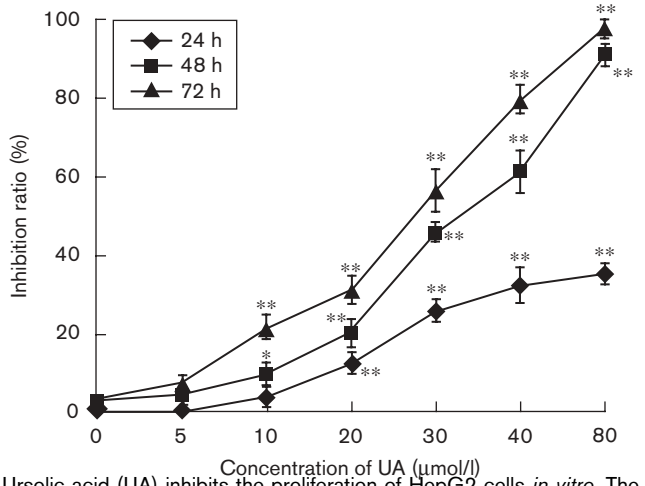
Results

Ursolic acid inhibits growth and induces apoptosis in HepG2 cells

We first tested the growth inhibitory effect of UA on HepG2 cells. As shown in Fig. 1, UA inhibited the proliferation of HepG2 cells in a concentration and time-dependent manner. Concentrations of UA required for 50% growth inhibition (IC_{50}) were 31.54 ± 2.12 µmol/l at 48 h and 26.65 ± 1.73 µmol/l at 72 h. After treatment with UA for 24 h, cells showed some morphological characteristics of apoptosis, including cell shrinkage, chromatin condensation, and the presence of apoptotic bodies (Fig. 2). Genomic DNA fragmentation and flow cytometry analysis further confirmed the operation of an apoptotic pathway in the cell death seen in response to UA. As shown in Fig. 3a, treatment with 20 and 30 µmol/l of UA for 24 h produced evident DNA laddering, a

typical subcellular symptom of apoptosis. Compared with control values (1.1%), HepG2 cells treated for 24 h with UA showed a concentration-dependent increase in the proportion of apoptotic cells (5.2, 14.8, and 24.9% for cells treated with 10, 20, and 30 μmol/l UA-treated HepG2 cells, respectively. $P < 0.05$ compared with control) at 24 h (Fig. 3b). These data provide evidence for the activation of apoptosis in UA-treated HepG2 cells.

Fig. 1



Ursolic acid (UA) inhibits the proliferation of HepG2 cells *in vitro*. The cells were seeded at 7.5×10^3 cells/well on 96-well plates and were then treated with the indicated concentrations of UA for 24, 48, and 72 h, respectively. Cytotoxicity was determined by the methyl thiazolyl tetrazolium assay. Cell viability was expressed as the percentage of cells untreated with UA, which served as the control group and was designated as 100%. The inhibition ratio was calculated from the loss of cell viability in cultures. Each point of the concentration response curve is the average of six wells. Results are expressed as mean ± SD. Significant differences between control and UA-treated group analyzed by Dunnett's test are indicated by asterisks: $^*P < 0.05$ and $^{**}P < 0.01$, $n = 3$.

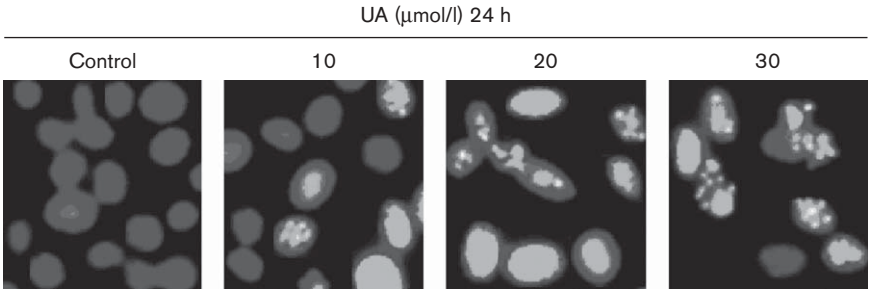
PI3K inhibition augments ursolic acid-induced apoptosis in HepG2 cells

The relationship between inhibition of PI3K/Akt pathway and UA-induced apoptosis can be seen in the apoptosis rate of HepG2 cells treated with a combination of UA and PI3K-specific inhibitor LY294002 for 24 h. As shown in Fig. 3b, LY294002 (20 μmol/l) treatment alone did not trigger obvious apoptosis (2.6%), and UA (10 μmol/l) treatment alone induced weak apoptosis (5.2%). However, a potent augmentation in apoptosis rate was observed in the combined treatment group (28.7%, $P < 0.01$ compared with 10 μmol/l UA treatment alone).

Modulation of the bcl-2 family and survivin by treatment with ursolic acid

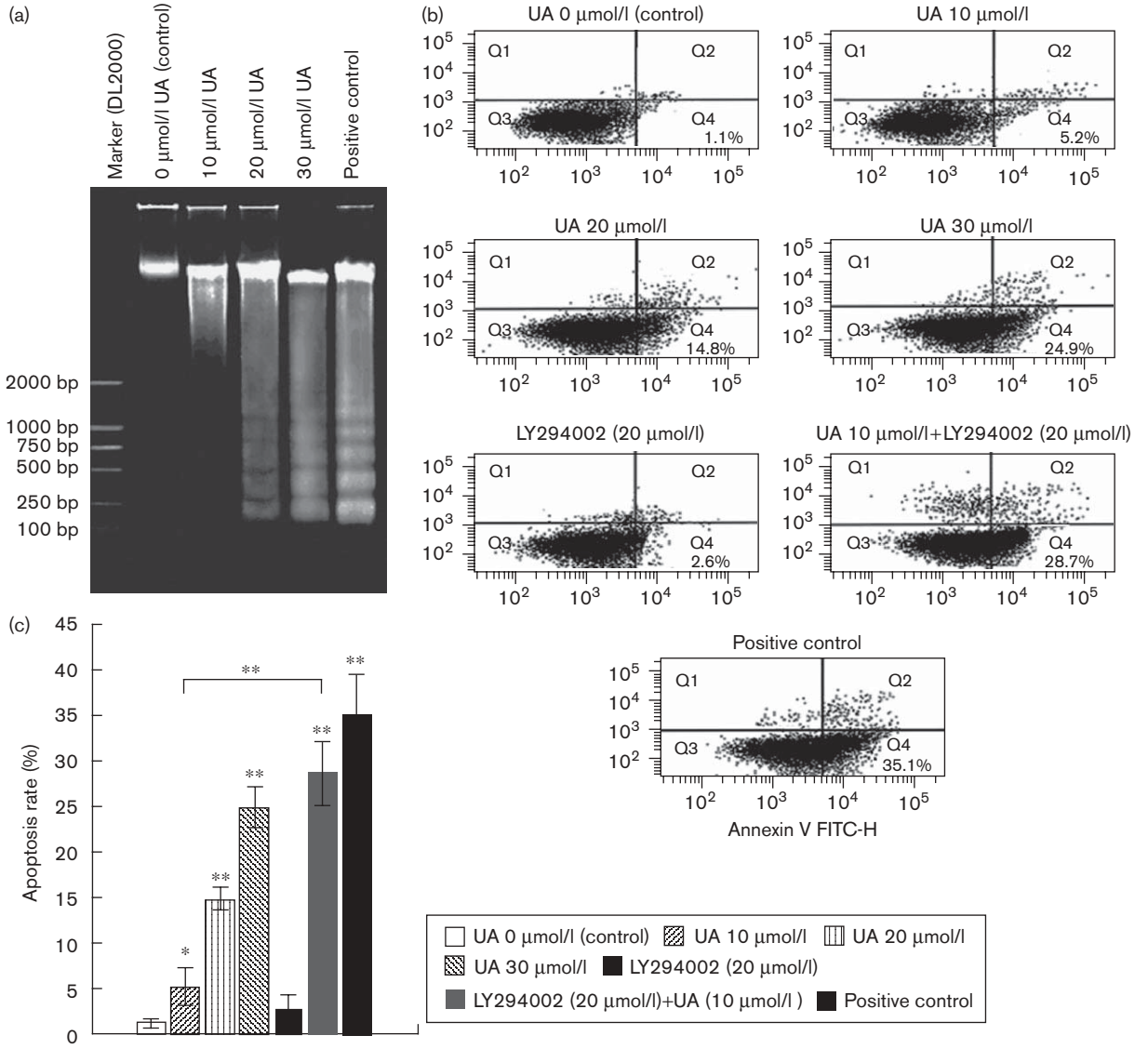
As shown in Fig. 4a, no obvious changes in p53 mRNA level were observed before 12 h. The mRNA level of bax was increased at 24 h in cells treated with 30 μmol/l UA, whereas bcl-2 and bcl-xL decreased significantly. This had the effect of greatly increasing the ratio of the proapoptotic bax to the antiapoptotic bcl-2 messenger (the bas/bcl-2 ratio was 8.74-fold higher than that of control for 30 μmol/l UA, $P < 0.01$). Treatment with UA also sharply decreased the survivin level in a time-dependent manner, only 18.2% of the survivin mRNA was expressed compared with control at 48 h ($P < 0.01$). Western blotting analysis (Fig. 4c) of cells treated with different concentrations of UA for 48 h indicated that the protein expression of bax and p53 increased in a concentration-dependent manner, in the group receiving 30 μmol/l UA, the levels of these two proteins were more or less doubled compared to control ($P < 0.01$). In contrast, considerable decreases in survivin, bcl-2, and bcl-xL protein levels were observed, which resulted in a significant increase in the bax/bcl-2 ratio in UA-treated HepG2 cells compared with control (8.31-fold higher than that of control for 30 μmol/l UA, $P < 0.01$). This was consistent with the corresponding change observed for mRNA expression at 48 h.

Fig. 2



Induction of nuclear condensation and apoptotic bodies by ursolic acid (UA), after incubation of cells in the presence of UA at indicated concentrations for 24 h. Cells were fixed with methanol for 5 min, washed with PBS twice, stained with 10 ng/ml Hoechst 33258 for 10 min at 37°C in darkness. Cells were then washed with PBS and examined under fluorescence microscopy (magnification, × 200). The results presented are representative of three independent experiments.

Fig. 3

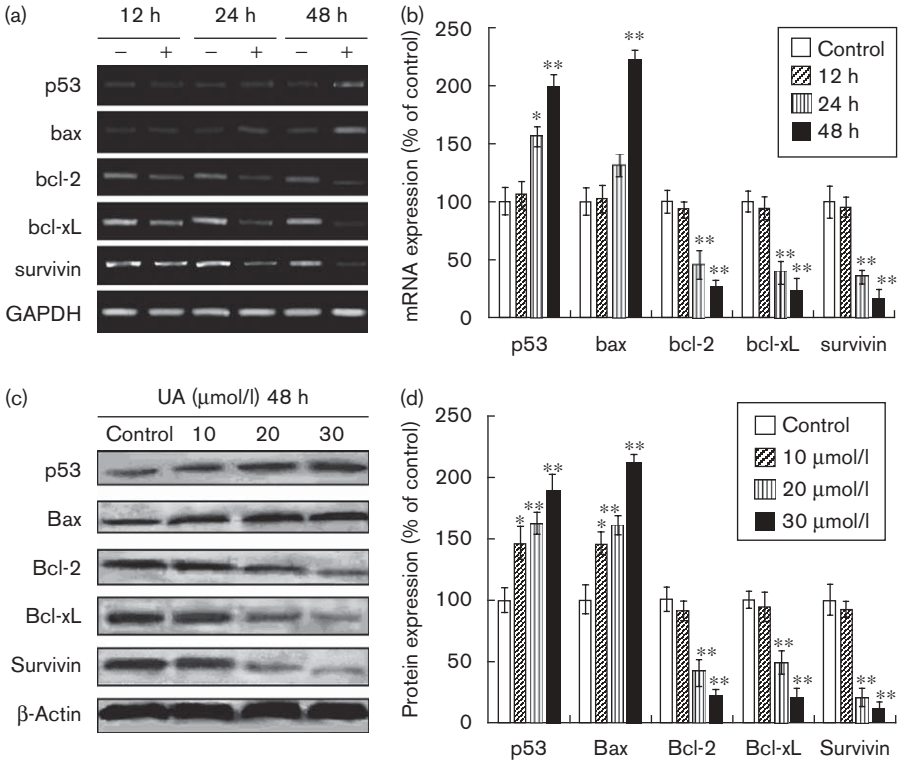


Ursolic acid (UA) treatment induces apoptosis in HepG2 cells. (a) Induction of DNA fragmentation by UA. After cells were treated with indicated concentrations of UA for 24 h, genomic DNA was extracted and determined using apoptotic DNA ladder kit. The results presented are representative of three independent experiments. (b) The extent of apoptosis was assessed by flow cytometry analysis. After incubating for 24 h in the presence of UA, LY294002, or a combination of UA and LY294002 at indicated concentrations, cells were collected and stained with annexin V-FITC/PI followed by flow cytometry analysis. Representative flow cytometry analysis scatter-grams of annexin V-FITC/PI-stained treatment showed four different cell populations; early apoptosis was shown by annexin V-FITC positively and PI negatively stained cells in the lower right section Q4 and 3 μmol/l of gambogic acid was used as the positive control. (c) The quantitative results of flow cytometry analysis. Error bars indicated the range of the determinations. Significant differences between control and UA-treated group analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$.

Cleavage of caspases in ursolic acid-treated HepG2 cells

As shown in Fig. 5a, after treatment with the indicated concentrations of UA for 48 h, significant increases in the active subunits of caspase-3, caspase-8, and caspase-9 were observed, and particularly for caspase-3 (4.86-fold, 9.01-fold and 10.11-fold more than control for treatments with 10, 20 and 30 μmol/l UA, respectively; $P < 0.01$). The use of specific fluorogenic peptide substrates to detect the activity changes of caspase-3, caspase-8, and caspase-9 in response to UA showed that the activity of caspase-8 and caspase-9 were similarly increased in a concentration-dependent manner (Fig. 5c). At the highest concentration tested, an almost seven-fold increase compared with control was detected ($P < 0.01$). UA induced a relatively higher rise in caspase-3 activity with an approximately 12-fold increase observed ($P < 0.01$ compared with

Fig. 4



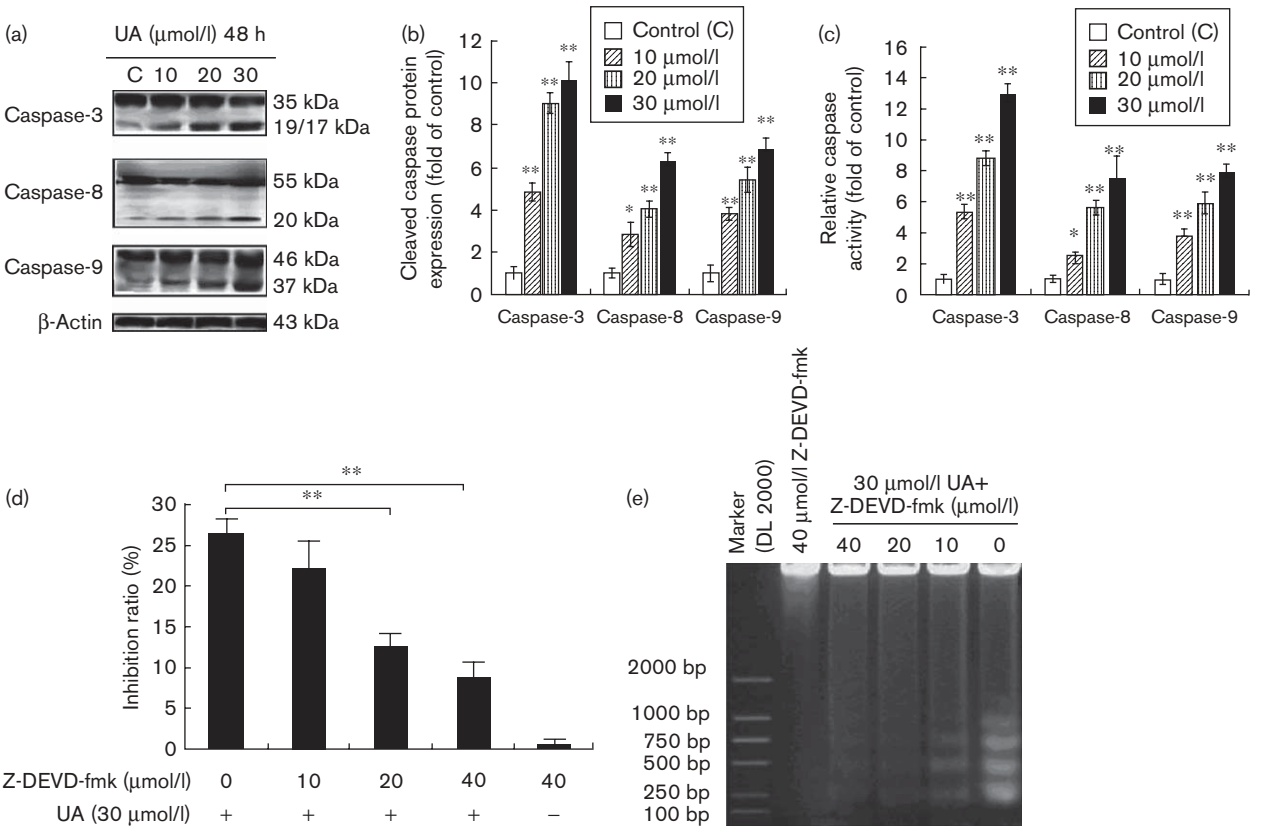
Ursolic acid (UA) affects the mRNA and protein expression of bcl-2 family and survivin in HepG2 cells. (a) UA suppresses the mRNA expression of bcl-2, bcl-xL and survivin, while increases p53 and bax levels in a time-dependent manner. HepG2 cells were treated with or without UA (30 μmol/l) for indicated periods, and total RNA was extracted and examined by reverse-transcriptase PCR. (b) The quantitative results of mRNA expression. Density value of each band was quantified using Quantity One software and expressed as a percentage of glyceraldehyde-3-phosphate dehydrogenase control. (c) Analysis of the protein expression of p53, bax, bcl-2, bcl-xL, and survivin in UA-treated cells. Cells were treated with different concentrations of UA for 48 h, collected, and lysates were subjected to western blotting with the appropriate antibodies. (d) The quantitative results of protein expression. Density value of each band was normalized to β-actin control and expressed as a percentage of control. Significant differences between control and UA-treated group analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$.

control). To confirm whether UA-induced apoptosis was caspase-3-dependent, caspase-3 inhibitor Z-DEVD-fmk was used to inhibit the activation of caspase-3. MTT assays indicated that Z-DEVD-fmk alone did not affect cell viability, but suppressed UA-reduced cell viability in a concentration-dependent manner (Fig. 5d). For the combined treatment of 40 μmol/l Z-DEVD-fmk and 30 μmol/l UA, the inhibition ratio was decreased to only 8.71% ($P < 0.01$ compared with 30 μmol/l UA alone). Z-DEVD-fmk also strongly inhibited UA-induced DNA fragmentation in a concentration-dependent manner, as shown in Fig. 5e, DNA laddering was hardly observed in the combined treatment of 40 μmol/l Z-DEVD-fmk and 30 μmol/l UA. These findings showed that the UA-induced apoptosis is mediated by caspase-3 in HepG2 cells.

Ursolic acid triggers apoptosis through the PI3K/Akt/survivin signal in HepG2 cells

As shown in Fig. 6a, after treatment with 30 μmol/l UA for 6 h, the level of PI3K/p85 was decreased to 90.9% of control, this trend continued during more prolonged treatments. The most significant reduction in PI3K level was observed at 12 h, as a decrease to 40.3% of control ($P < 0.01$). Treatment with UA also inhibited the phosphorylation of Akt in HepG2 cells. After 12 h of treatment, the phospho-Akt level was dramatically decreased to 46.3% of control in response to 30 μmol/l UA, whereas the total Akt level remained unchanged throughout the course of the experiment. By 24 h, survivin expression was greatly decreased by UA treatment. To further clarify whether the PI3K/Akt pathway is involved in regulating survivin, we detected the change of survivin expression in the presence of PI3K-specific inhibitor LY294002. Results showed that LY294002 suppressed the expression of survivin in a concentration-dependent manner, 30 μmol/l LY294002 treatment potently downregulated survivin level (Fig. 6b). As indicated in Fig. 6c, LY294002 (10 μmol/l) and UA (10 μmol/l) synergistically decreased survivin level. When applied singly at this concentration, LY294002 and UA had only weak effects on survivin (95.32 and 86.17% of control, respectively), whereas in combination, these exerted

Fig. 5



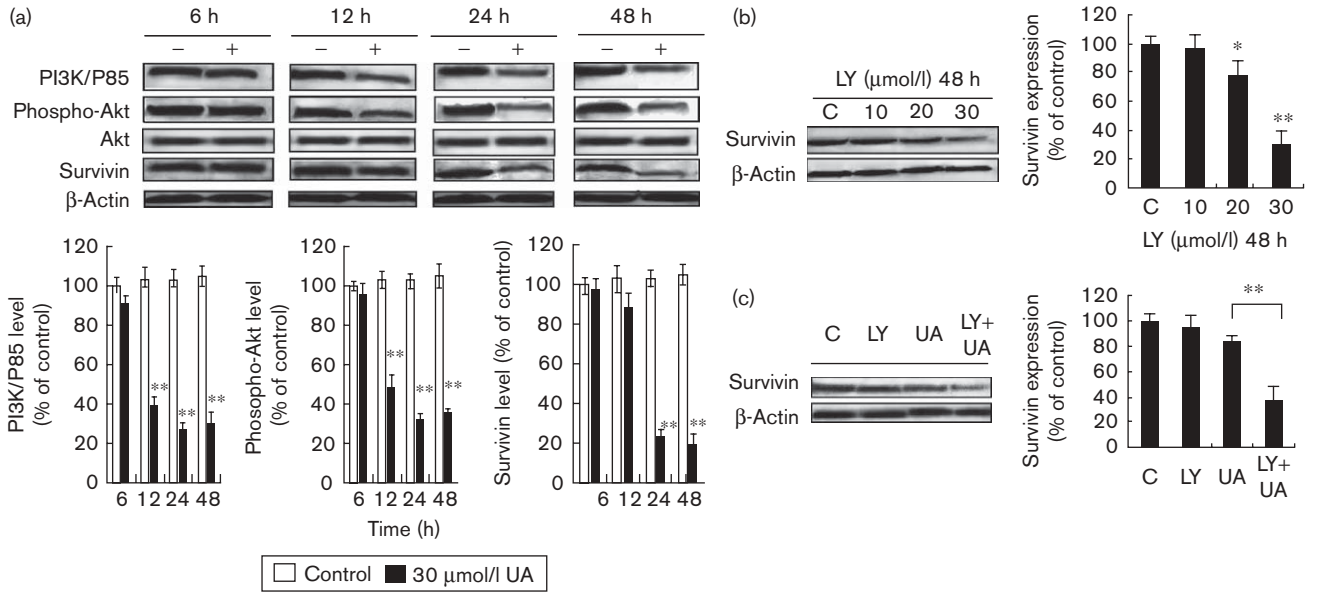
Induction of ursolic acid (UA) on the expression and activity of caspases. (a) UA increases the protein levels of caspase-3, caspase-8, and caspase-9. Cells were incubated with different concentrations of UA for 48 h, collected, and lysates were subjected to western blotting with the appropriate antibodies against caspase-3, caspase-8, and caspase-9. (b) The quantitative results of caspases expression. Density value of each band was normalized to β-actin control and expressed as fold of control. (c) Induction of caspases activity after treatment with UA. After treatment with different concentrations of UA for 48 h, caspases activity was assessed by a spectrofluorimeter as described in 'Materials and Methods'. Data were expressed relative to the control whose activity was considered as 1. Significant differences between control and UA-treated group analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$. C, control. (d)(e) caspase-3-specific inhibitor Z-DEVD-fmk inhibits the cytotoxic effect and DNA fragmentation induced by UA. After co-incubation with different concentrations of caspase-3 inhibitor Z-DEVD-fmk and 30 μmol/l UA for 24 h, cells were subjected to methyl thiazolyl tetrazolium assay and DNA fragmentation analysis. Significant differences between combined treatment group and UA-treated group were analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$.

a significant reduction in survivin level (only 39.01% of control, $P < 0.01$ compared with 10 μmol/l UA alone). Together with the results of flow cytometry analysis, a significant role for the PI3K/Akt signaling pathway was indicated for UA-induced apoptosis and survivin regulation.

Discussion

Apoptosis is an activated cellular death process that is induced by physiological or pathological factors to eliminate of redundant and damaged cells. Apoptotic cells undergo a variety of biochemical and morphological events that include cell shrinkage, mitochondrial degradation, and nuclear DNA fragmentation [15]. All these processes involve changes in the expression and activity of many genes. Bcl-2 is a prooncogene that encodes a 26 kDa mitochondrial-associated protein responsible for inhibiting cytochrome *c* translocation from the mitochondria to cytoplasm and that blocks the caspase activation step of the mitochondrial intrinsic apoptotic pathway [24,25]. The tumor suppressor gene p53 and death-promoting members of the bcl-2 family are important regulators in the process of apoptosis. Upregulated p53 directly promotes bax expression that changes the integrity of the mitochondria to induce apoptosis [26]. In this study, treatment with UA inhibited growth in a concentration and time-dependent manner by inducing apoptosis in HepG2 cells. The expression of p53 and bax proteins was increased in UA-treated HepG2 cells, whereas that of bcl-2 and bcl-xL was simultaneously decreased. This had the effect of greatly altering the ratio of the proapoptotic bax to the antiapoptotic bcl-2. All of these effects of UA treatment are known to increase the mitochondrial membrane penetrability, resulting in the release of cytochrome *c* from mitochondria into cytoplasm, and the triggering of apoptosis.

Fig. 6



(a) Ursolic acid (UA) regulates the protein level of PI3K, phospho-Akt, and survivin in time order. HepG2 cells were treated without or with 30 μmol/l UA for 6, 12, 24, and 48 h, respectively. Cell lysates were prepared and subjected to western blotting with appropriate antibodies against PI3K/p85, phospho-Akt (phosphorylated at Ser473), total Akt protein and survivin. Density value of each band was normalized to β-actin control and expressed as percentage of control. (b) PI3K inhibition decreases survivin expression. After treatment with different concentrations of LY294002 for 48 h, cell lysates were prepared and subjected to western blotting with anti-survivin antibody. Density value of each band is normalized to β-actin control and expressed as percentage of control. Significant differences between control and UA-treated or LY294002-treated group analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$. C, control; LY, LY294002. (c) PI3K inhibition enhances UA-reduced survivin expression. After incubating for 48 h in the presence of UA (10 μmol/l), LY294002 (10 μmol/l), or a combination of 10 μmol/l UA and 10 μmol/l LY294002, cell lysates were prepared and subjected to western blotting with the antibody against survivin. Density value of each band was normalized to β-actin control and expressed as percentage of control. Significant differences between combined treatment group and UA-treated group analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$. C, control; LY, LY294002.

Caspases, particularly caspase-3, are central proteases for apoptosis [27]. Caspases are synthesized as proenzymes and are then activated by proteolysis at several sites. An *N*-terminal peptide is excised and the proenzyme is separated into large and small subunits. The active caspase is a heterotetramer of two large and two small subunits [28]. In this study, a significant increase in the expression and activity of active caspase-3 was observed after UA treatment. In addition, the caspase-3 inhibitor Z-DEVD-fmk significantly inhibited both the cytotoxic effect and the DNA fragmentation induced by UA, showing the involvement of caspase-3 in UA-induced apoptosis. The cytotoxic effects of UA, however, were not completely prevented by Z-DEVD-fmk, which provides evidence that factors in addition to caspase-3 are also involved. Survivin plays an important role in the regulation of cell death and cell survival. Although absent from fully differentiated adult human tissues, it is overexpressed in transformed cell lines, in cancers *in vivo*, and in fetal tissues [29]. Survivin has been characterized as a multifunctional protein that suppresses apoptosis and cell division [30]. Many investigators suggest that survivin inhibition may be an important strategy for the treatment of cancer [31]. Survivin can bind to and potently inhibit caspases, including caspase-3 [18], which suggests that the potent inhibition of survivin expression by UA could augment the activity of caspase-3, thus substantially enhancing UA-induced apoptosis in HepG2 cells. Consequently, UA may induce apoptosis in HepG2 cells as a result of downregulation of survivin and subsequent activation of caspase-3.

The PI3K/Akt pathway regulates a wide range of cellular processes, including cell survival, cellular apoptosis, and cytoskeletal rearrangement [20]. As an important downstream target of the PI3K/Akt pathway, serine-threonine kinase Akt is activated by direct contact of its plectstrin homology domain with PIP_3, and phosphorylation at Thr308 and Ser473. Through interaction with downstream effectors such as bcl-2 family, phospho-Akt then transfers a survival signal to cells [32]. This study sought to determine the effects of PI3K/Akt on UA-induced apoptosis and the expression of survivin. Our results showed that the inhibition of PI3K/Akt pathway by LY294002 significantly increased UA-induced apoptosis, suggesting that UA-induced apoptosis is associated with the inactivation of PI3K/Akt. Initial kinetic experiments indicated that survivin level was greatly decreased

after the maximal decrease of PI3K/p85 and phospho-Akt levels. Further results also confirmed that LY294002 not only downregulated survivin expression alone, but also significantly enhanced the reduction in survivin expression caused by UA. Furthermore, survivin appears to be a downstream target of PI3K/Akt pathway [33]. All these findings suggest that the downregulation of survivin observed in this study is mediated by inactivating PI3K/Akt in UA-treated HepG2 cells. However, further study is needed to define the mechanism underlying the downregulation of survivin by PI3K/Akt inhibition.

Taken together, the results of this study indicate that UA downregulates survivin, but active caspases are also involved in the signal transduction pathway leading to UA-induced apoptosis, which involves changes in p53, bax, and bcl-2 expression. The inhibition of the PI3K/Akt pathway by UA may be a means of downregulating survivin expression, a downstream target of the PI3K/Akt pathway. Suppression of survivin expression, through inactivation of PI3K/Akt by UA, may contribute to the subsequent activation of caspase-3-mediated apoptosis in HepG2 cells. Inhibition of the PI3K/Akt/survivin pathway by compounds such as UA may therefore provide a novel strategy for the treatment of hepatocellular carcinoma.

In addition, accumulating evidence about the safety of UA has shown that UA has no inhibitory effects on primarily cultured normal mouse hepatocytes. Furthermore, it may also have beneficial effects, as UA has been reported to have a protective effect on ethanol-mediated experimental liver damage in rats [34,35]. Recently, Chadalapaka *et al.* [36] have modified the chemical structure of UA and have obtained 2-substituted UA derivatives with substantially higher biological activity. All of these studies indicate that UA may have a promising future in the research and development of clinical antihepatoma drugs with high anticancer activity and low nontarget toxicity.

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