Cross-talk between PI3K/Akt and MEK/ERK pathways mediates endoplasmic reticulum stress-induced cell cycle progression and cell death in human hepatocellular carcinoma cells

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Abstract. The unfolded protein response (UPR) is a conserved adaptive response utilized by cells to cope with endoplasmic reticulum (ER) stress. In addition to the UPR, cells also trigger other adaptive responses under ER stress conditions. Although the PI3K/Akt and MEK/ERK pathways are known to protect cells from ER stress-induced apoptosis, their other functions under ER stress remain elusive. Here, we showed that long-term ER stress resulted in inactivation of Akt and activation of ERK in human hepatocellular carcinoma (HCC) cells. Importantly, both PI3K inhibitor LY294002 and dominant-negative Akt construct promoted tunicamycin- and thapsigargin-induced ERK phosphorylation. In addition, constitutively active Akt construct inhibited ER stress-induced ERK phosphorylation. We also showed that ER stress-induced PI3K/Akt inactivation contributed to cell cycle arrest and MEK/ERK inhibition moderately increased cell percentage in the S phase. It is notable that U0126 made HCC cells much more sensitive to ER stress-induced apoptosis than LY294002. Taken together, our results indicate that there is cross-talk between the PI3K/Akt and MEK/ERK cascades under ER stress in HCC cells, which contributes to both cell cycle arrest and cell survival. We propose that ER stress-induced cross-talk between the PI3K/Akt and MEK/ERK cascades is a protective mechanism utilized by HCC cells to adapt to stress.

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Abbreviations: HCC, human hepatocellular carcinoma; UPR, the unfolded protein response; ER, endoplasmic reticulum; ATF6, activating transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, PKR-like ER kinase; GRP78, 78 kDa glucose-regulated protein; eIF2α, eukaryotic translation initiation factor 2α; CKIs, CDK inhibitors; CHOP, C/EBP homologous protein

Key words: PI3K/Akt, MEK/ERK, cross-talk, endoplasmic reticulum stress, cell cycle, apoptosis, human hepatocellular carcinoma

Introduction

In eukaryotic cells, most secreted and transmembrane proteins fold and mature in the lumen of the endoplasmic reticulum (ER). The ER relies on multiple resident chaperone proteins, a high level of calcium and an oxidative environment to facilitate protein folding and transport synthesized proteins efficiently (1). The perturbation of ER functions, including disruption of Ca^{2+} homeostasis, inhibition of protein glycosylation or disulfide bond formation, hypoxia and virus or bacteria infection, causes accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved response, termed the unfolded protein response (UPR) (2-4). The canonical mammalian UPR pathway includes three branches, where the transmembrane proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and PKR-like ER kinase (PERK) serve as proximal sensors (5). Under unstressed conditions, the luminal domains of these sensors are occupied by the glucose-regulated protein (GRP78), which represses these signaling pathways. Upon ER stress, sequestration of GRP78 by unfolded proteins activates these sensors, inducing the UPR (6). Acting as a cytoprotective response, the UPR enables the cell to reduce the accumulation of unfolded proteins and restore normal ER functions through transcriptional induction of UPR genes, translational attenuation of global protein synthesis and ER-associated protein degradation (3,6-8). However, if ER homeostasis cannot be restored, prolonged UPR switches from pro-survival to pro-apoptotic and the cells are destroyed by apoptosis.

During the UPR, PERK-dependent eukaryotic translation initiation factor 2α (eIF2α) phosphorylation induces a global down-regulation of protein synthesis (2). The protein synthesis attenuation is coordinated with G_1 phase arrest, which likely serves as a stress-induced response that allows cells to re-establish ER homeostasis (2,9,10). Progression through G_1 phase requires one or more of the D-type cyclins (D1, D2, or D3) activation in association with either CDK4 or CDK6, followed by cyclin E- and A-dependent kinase CDK2 activation (11). Cell cycle arrest can be achieved through degradation of cyclin subunits, specific posttranslational modifications of the CDK subunits, or association of active cyclin bound CDKs with polypeptide CDK inhibitors (CKIs) (12,13). An important consequence of ER stress-induced translation attenuation is the loss of cyclin D1, which plays a

pivotal role in G_1 phase arrest under ER stress conditions (10).

The PI3K/Akt and MEK/ERK signal transduction cascades are pivotal in transmitting signals from membrane receptors to downstream targets that regulate critical cellular responses, such as proliferation, apoptosis, differentiation and senescence (14). Previous evidence indicated that the PI3K/Akt and MEK/ERK pathways play critical roles in controlling cell survival by suppressing ER stress-induced cell death (15). The important functions of the PI3K/Akt and MEK/ERK cascades in cell cycle and apoptosis regulation have been extensively studied. Therefore, we hypothesized that the PI3K/Akt and MEK/ERK cascades might play some roles in the cell cycle regulation as well as in anti-apoptosis under ER stress. In this study, we demonstrate a functional cross-talk between the PI3K/Akt and MEK/ERK pathways in human hepatocellular carcinoma (HCC) cells under ER stress and this cross-talk regulates both the cell cycle progress and cell survival.

Materials and methods

Materials. Thapsigargin (TG) and tunicamycin (TU), were purchased from Sigma Chemical Company. The PI3K inhibitor LY294002 and MEK-specific inhibitor U0126 were purchased from Merck Chemicals. The antibodies against cyclin D1 and GAPDH were purchased from Santa Cruz Biotechnology. The antibodies against HA-tag, phospho-Akt (Ser473), Akt, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-c-raf (Ser259) and c-raf were purchase from Cell Signaling Technology. Secondary antibodies were purchased from Santa Cruz Biotechnology.

Cell culture and treatment. Human hepatocellular carcinoma cell lines SMMC-7721, Hep3B and HepG2 were maintained at 37˚C in a humidified incubator containing 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Tunicamycin and thapsigargin were used to induce ER stress response.

Transfections. The expression vectors for constitutively active Akt (myr-HA-Akt) and domain-negative HA-Akt (K179M) were kindly provided by Professor Jin Q. Cheng. HA-cyclin D1 overexpression vector was kindly provided by Professor Mark E. Ewen. SMMC-7721 cells were transfected by polyethylenimine (PEI) with plasmids coding for the neomycin-resistance gene alone (pcDNA3.1) or with HA-cyclin D1-pcDNA3.1 construct. Stable clones were selected in the presence of 300 μg/ml Geneticin (G418). Transient transfection of pcDNA3.1, HA-myr-Akt or HA-AKT (K179M) constructs into SMMC-7721 and Hep3B ($4x10^5$ cells/well) cells were performed using PEI and GenePorter transfection reagent, respectively.

Western blot analysis. Cells were lysed in Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 5 mg/ml aprotinin, 20 mM leupeptin and 1 mM sodium orthovanadate) and centrifuged at 12,000 x g for 15 min. Protein concentrations were measured using the BCA assay (Santa Cruz Biotech). Proteins were applied to SDS-PAGE. After electrophoresis, proteins were blotted to polyvinylidene fluoride (PVDF) membranes and then blocked with 5% skim milk powder with 0.1% Tween-20. The blots were then probed at 4˚C overnight with relevant antibodies, washed by TBST (TBS containing 0.1% Tween-20) three times and probed with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Immunoreactive material was detected using the ECL kit according to manufacturer's instruction (Santa Cruz Biotech).

Cell cycle analysis. Cells growing exponentially in 6-well plates were synchronised (G_0/G_1) by placing in serum-free DMEM for 20 h before treated with tunicamycin and thapsigargin for the indicated time period, then fixed with ethanol (70%) at 4˚C overnight, the cells were washed with PBS and stained with 50 mg/ml propidium iodide (PI), which stains DNA, containing 200 mg/ml RNase for 30 min at 37˚C. The fluorescence volume of PI was measured by flow cytometry and the percentage of cells in each phase of the cell cycle was calculated. The experiments were repeated three times.

Apoptosis analysis. Cells were treated with tunicamycin and thapsigargin for the indicated time. Apoptosis was detected using Annexin V-FITC apoptosis detection kit (PharMingen) according to the manufacturer's manual. Annexin V staining was analyzed by flow cytometry within 1 h. The experiments were repeated three times.

Statistical analysis. Values presented are expressed as mean ± standard deviation. After acquiring data, the Tukey-Kramer comparison test was applied to determine statistical significance. A value of $p<0.05$ was considered to be statistically significant.

Results

ER stress induces cell cycle arrest in HCC cells. To evaluate the impact of ER stress on the cell cycle progress in HCC cells, ER stress inducers, tunicamycin that directly inhibits N-linked glycosylation and thapsigargin that inhibits Ca^{2+}-ATPase of the ER and induces the perturbation of calcium homeostasis in ER, were used to induce activation of the UPR in SMMC-7721, Hep3B and HepG2 cells. Tunicamycin markedly up-regulated GRP78 protein expression, indicative of activation of the UPR, in SMMC-7721, Hep3B and HepG2 cells at indicated time points (Fig. 1A). The effects of tunicamycin and thapsigargin on the cell cycle progress in SMMC-7721, Hep3B and HepG2 cells were detected by flow cytometry (FCM). Tunicamycin induced an increase of the percentage of G_0/G_1 phase cells, representing G_1 arrest of the cell cycle, in all of three cell lines in a concentration- and time-dependent manner (Fig. 1B and C). Thapsigargin also induced G_1 phase arrest in a concentration- and time-dependent manner in HCC cells (data not shown). These data suggest that ER stress induces cell cycle arrest in HCC cells.

Cyclin D1 loss is required for ER stress-induced G_1 phase arrest. As PERK/eIF2α, which is one specific branch of the

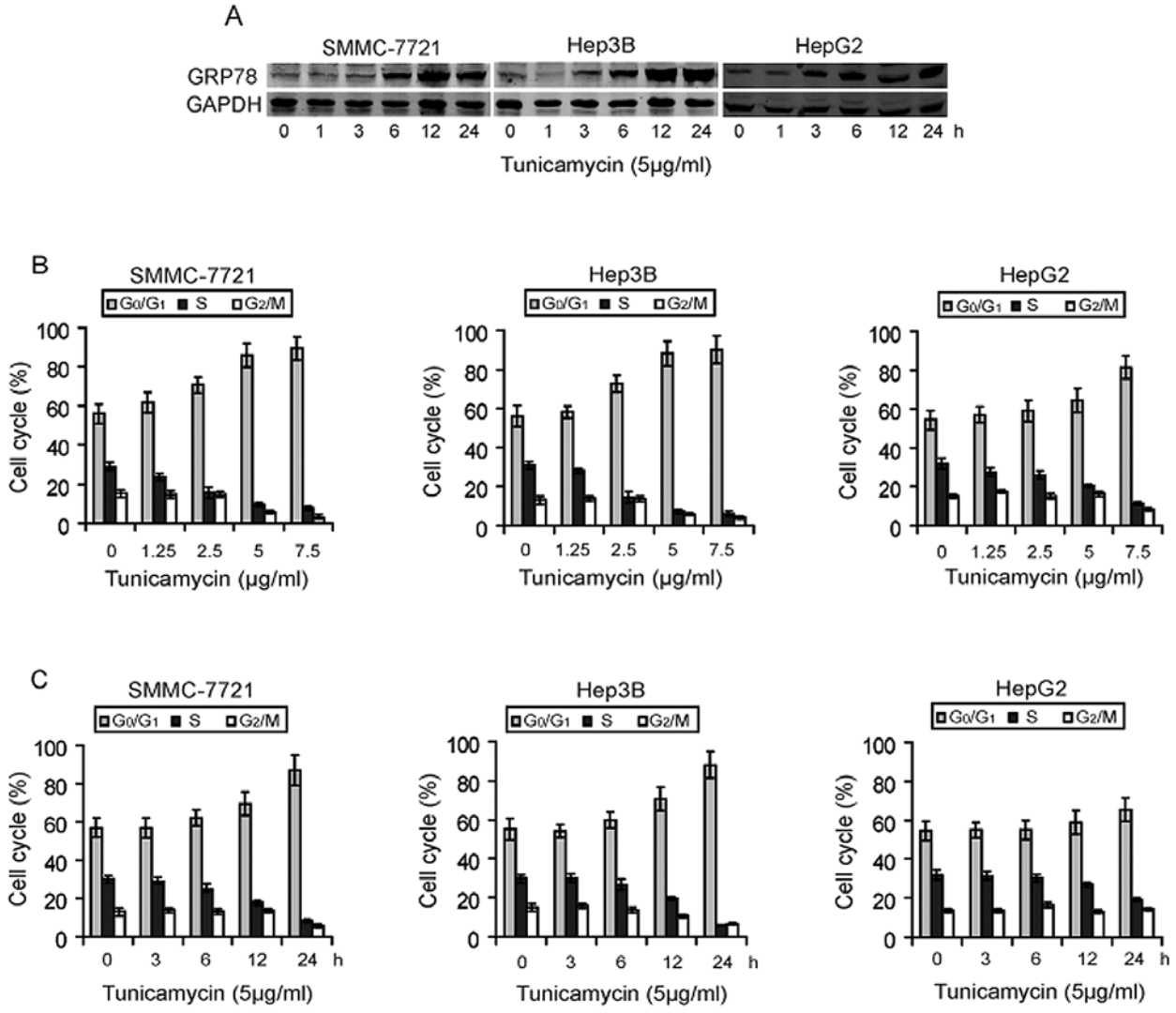

Figure 1. Tunicamycin induces cell cycle arrest in HCC cells. (A) Tunicamycin induces activation of the UPR in HCC cells. Whole-cell lysates from SMMC-7721, Hep3B and HepG2 cells with tunicamycin (5 μg/ml) treatment for indicated periods were subjected to Western blot analysis of GRP78 expression. GAPDH was used as loading control. (B) Tunicamycin induces cell cycle arrest in HCC cells in a concentration-dependent manner. SMMC-7721, Hep3B and HepG2 cells with tunicamycin treatment for 24 h at indicated dose were subjected to measurement of cell cycle distribution using flow cytometry after staining with propidium iodide. Data are presented as mean values ± SD of three measurements. (C) Tunicamycin induces cell cycle arrest in HCC cells in a time-dependent manner. SMMC-7721, Hep3B and HepG2 cells with tunicamycin (5 μg/ml) treatment for indicated periods were subjected to measurement of cell cycle distribution using flow cytometry after staining with propidium iodide. Data are presented as mean values ± SD of three measurements.

UPR, mediates global translational repression after treatment of cells with ER stress inducers (2), we then tested whether the UPR inhibited cyclin D1 accumulation in HCC cells after tunicamycin treatment. Western blot analysis revealed that a significant decrease in protein levels for cyclin D1 in SMMC-7721, Hep3B and HepG2 cells after tunicamycin treatment (Fig. 2A). To determine the role of cyclin D1 loss in ER stress-induced G_1 phase arrest in HCC cells, cyclin D1 stable over-expressed SMMC-7721 cells and the control cells (Fig. 2C) were treated with tunicamycin or thapsigargin for indicated time period. Enforced cyclin D1 overexpression resulted in reduction in the fraction of G_1 phase cells, with a concomitant increase in the percentage of G_2 phase cells in tunicamycin-treated SMMC-7721 cells (Fig. 2B). Similar results were obtained in thapsigargin-treated cyclin D1 stable overexpressed SMMC-7721 cells (data not shown). These results not only indicate that cyclin D1 loss is required for ER stress-induced G_1 phase arrest, but also imply that other mechanisms are involved in cell cycle progress regulation in HCC cells under ER stress.

ER stress induces the PI3K/Akt and MEK/ERK pathway activation with different kinetics. As the PI3K/Akt and MEK/ERK are critical pathways in regulating cell cycle and cell survival, we investigated the phosphorylation levels of Akt and ERK under ER stress using antibodies, which recognize phosphorylated Akt and ERK, respectively. The results showed that the Akt phosphorylation levels were up-regulated within 3 h and down-regulated from 3 to 24 h in SMMC-7721 (Fig. 3A) and Hep3B (Fig. 3B) cells, when exposed to tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l). In contrast, the total Akt protein expression was not changed in response to ER stress at indicated time points (Fig. 3A and B), indicating that the changes in Akt phosphorylation was not due to the changes in total Akt protein. Furthermore, we found that

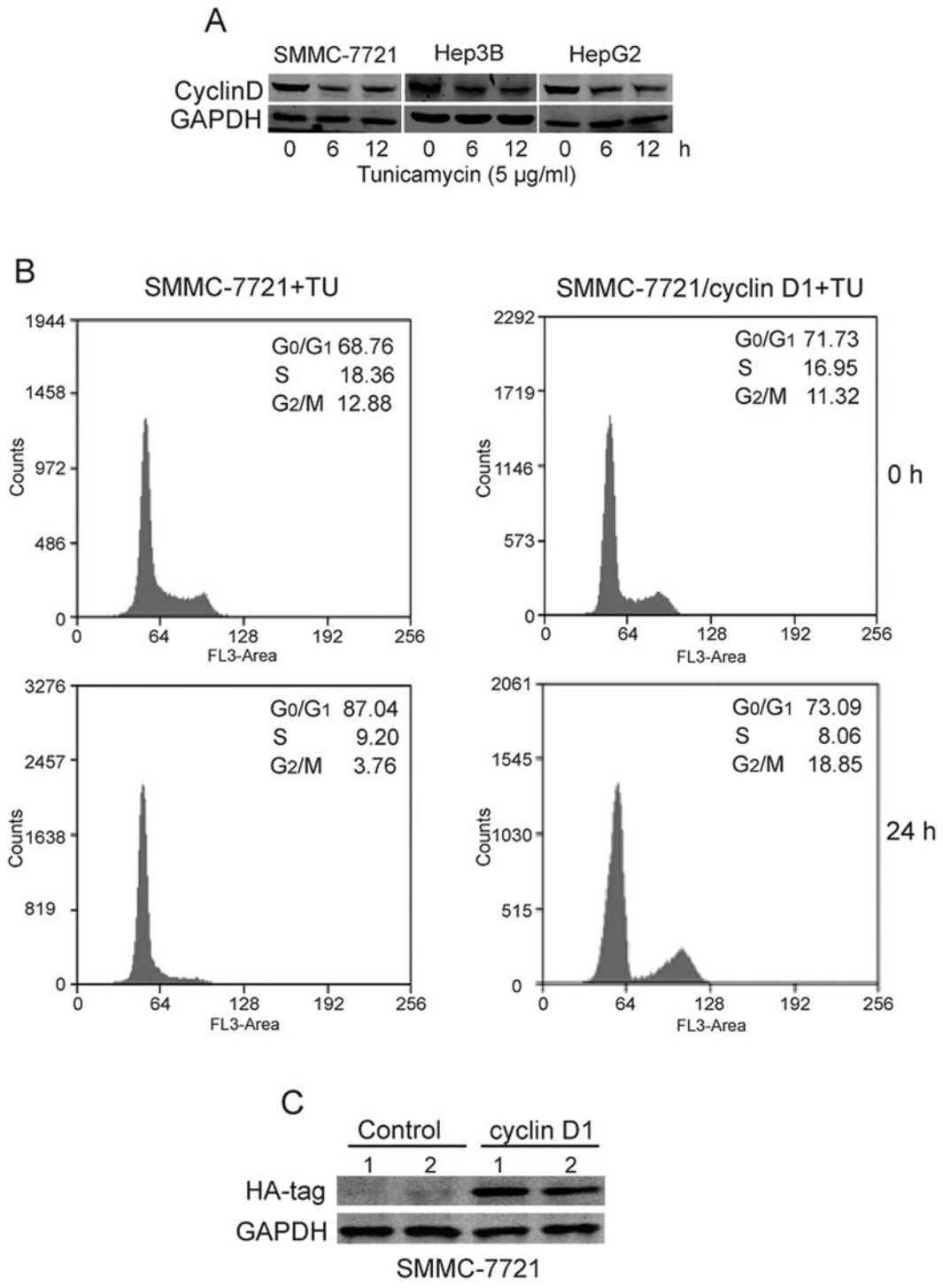

Figure 2. Overexpression of cyclin D1 induces reduction in G_0/G_1 phase cells and increase in G_2/M phase cells. (A) Tunicamycin induces cyclin D1 loss in HCC cells. Whole-cell lysates from SMMC-7721, Hep3B and HepG2 cells with tunicamycin (5 μg/ml) treatment for indicated periods were subjected to Western blot analysis of cyclin D1 expression. GAPDH was used as loading control. (B) Stable overexpression of cyclin D1 decreases the fraction of G_1-phase cells under ER stress. Cyclin D1 stable expressed SMMC-7721 cells were subjected to measurement of cell cycle distribution using flow cytometry after staining with propidium iodide when treated with tunicamycin (5 μg/ml) for indicated periods. Data are representative of three individual experiments. (C) Whole-cell lysates from cyclin D1 stable expressed SMMC-7721 cells and the control cells were collected for Western blot analysis.

long-term exposure to tunicamycin or thapsigargin resulted in ERK1/2 phosphorylation increases in SMMC-7721 (Fig. 3A) and Hep3B (Fig. 3B) cells without altering total amounts of ERK protein. These results indicate that ER stress induced Akt and ERK activation with different kinetics in HCC cells. On the contrary, insulin (100 nmol/l) induced both Akt and ERK long-lasting (24 h) activation in SMMC-7721 and Hep3B cells (Fig. 3C), indicating that ER stress induces the PI3K/Akt and MEK/ERK pathway activation with different kinetics specifically.

PI3K/Akt inactivation mediates ER stress-induced ERK activation. To study the potential cross-talk between the PI3K/Akt and MEK/ERK pathways under ER stress, we investigated the

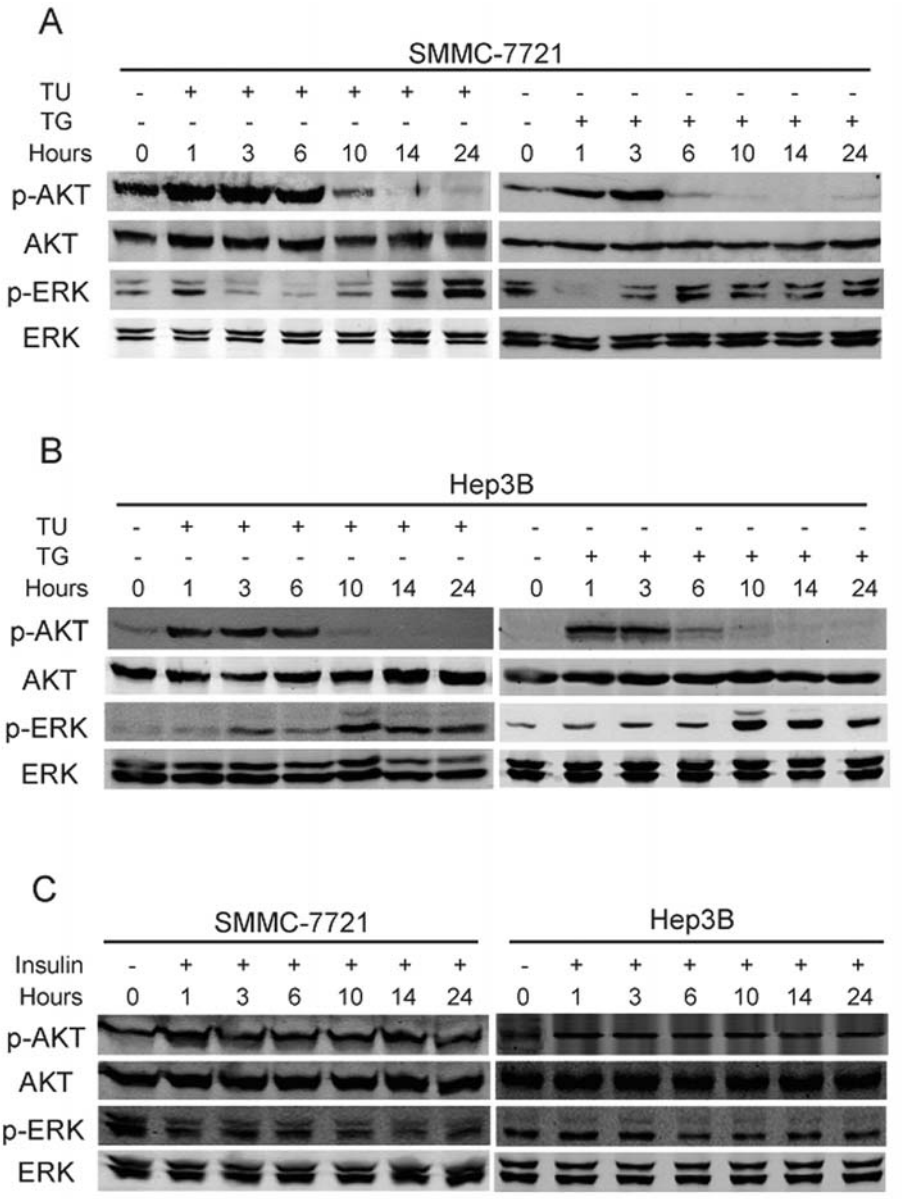

Figure 3. ER stress induces the PI3K/Akt and MEK/ERK pathway activation with different kinetics. (A and B) Tunicamycin and thapsigargin induce different kinetics of the PI3K/Akt and MEK/ERK activation in SMMC-7721 (A) and Hep3B (B) cells. Whole-cell lysates from SMMC-7721 and Hep3B cells with tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l) treatment for indicated periods were subjected to Western blot analysis of phosphorylated Akt and ERK. (C) Insulin induces both Akt and ERK long-lasting activation in SMMC-7721 and Hep3B cells. Whole-cell lysates from SMMC-7721 and Hep3B with insulin (100 nmol/l) treatment for indicated periods were subjected to Western blot analysis of phosphorylated Akt and ERK.

effect of PI3K/Akt inhibition on ERK activation. SMMC-7721 and Hep3B cells were treated with PI3K inhibitor LY294002 1 h before the addition of tunicamycin or thapsigargin for indicated period. Our results showed that LY294002 not only efficiently blocked Akt activation but also markedly increased ERK phosphorylation under ER stress in SMMC-7721 and Hep3B (Fig. 4A) cells. The PI3K inhibitor LY294002 alone did not cause ERK phosphorylation increase. In contrast, Western blotting showed that MEK inhibitor U0126 efficiently inhibited ERK activation, but had no effect on tunicamycin- or thapsigargin-induced Akt activation (Fig. 4B). Thus, it is suggested that ER stress-induced Akt activation is mediated through PI3K and the PI3K/Akt pathway inactivation is involved in increased ERK activity.

To confirm the effect of the PI3K/Akt pathway on the MEK/ERK pathway activation under ER stress, SMMC-7721 and Hep3B were transiently transfected with constitutively activated Akt mutant construct (HA-myr-Akt), which is constitutively targeted to the plasma membrane. The data showed that HA-myr-Akt markedly inhibited ERK phosphorylation under ER stress in SMMC-7721 and Hep3B (Fig. 4C) cells. Furthermore, transient transfection of the dominant-negative Akt (K179M) construct resulted in up-regulation of ERK phosphorylation in SMMC-7721 and Hep3B cells under ER stress (data not shown). These results support the ideal that ER stress-induced Akt inactivation mediates ERK activation in HCC cells.

As Akt can phosphorylate c-raf on Ser259 and lead to the Raf/MEK/ERK cascade inactivation (16,17), we studied whether the PI3K/Akt pathway might regulate the phosphorylation of c-raf under ER stress in HCC cells. The results showed that PI3K inhibitor LY294002 decreased the phospho-

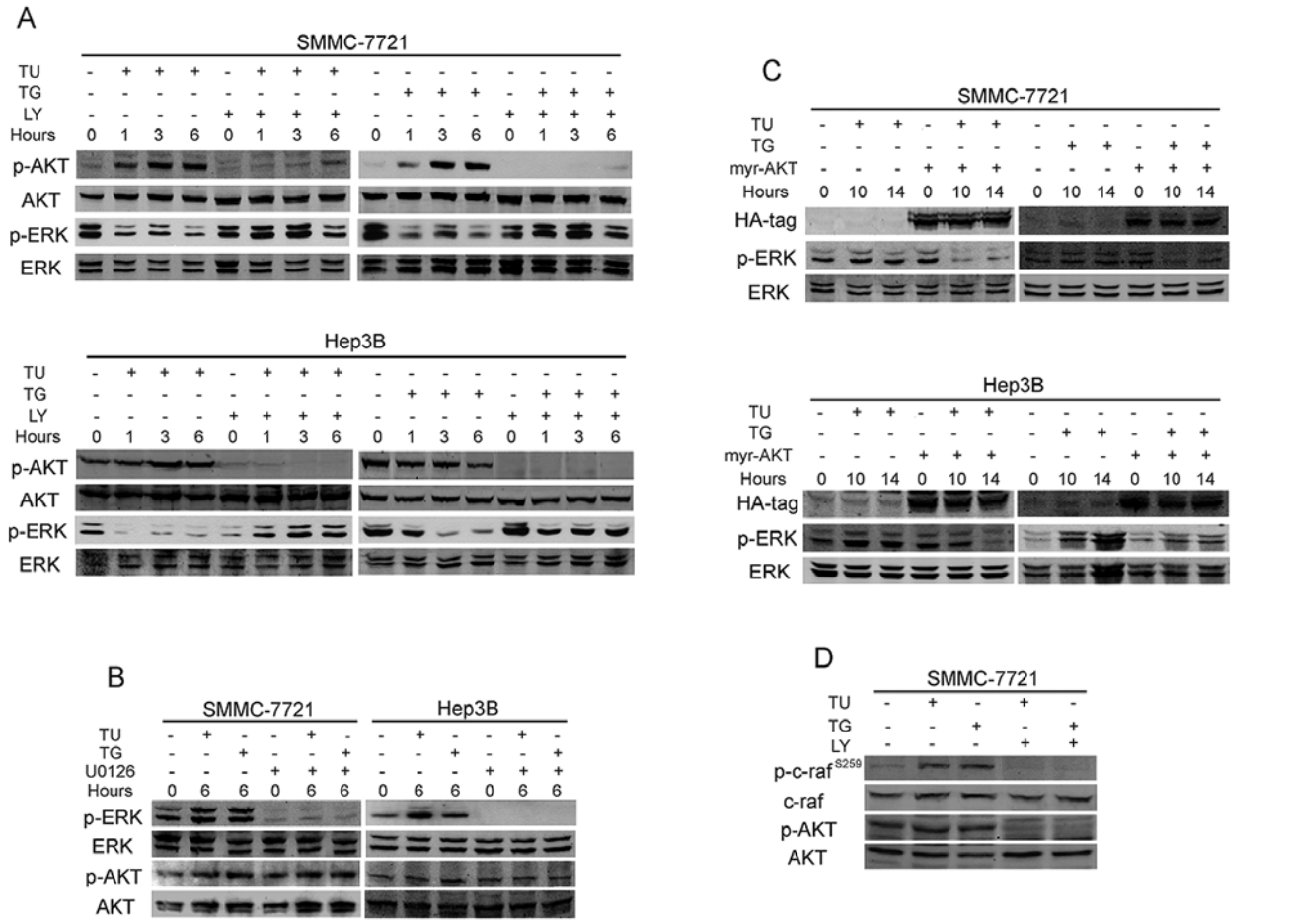

Figure 4. PI3K/Akt inactivation mediates ER stress-induced ERK activation. (A) PI3K inhibitor LY294002 increases tunicamycin- and thapsigargin-induced ERK phosphorylation. SMMC-7721 (upper panel) and Hep3B (bottom panel) cells were treated with tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l) for indicated periods with or without LY294002 (30 μmol/l) pre-incubation for 1 h. Whole-cell lysates were subjected to Western blot analysis of phosphorylated Akt and ERK. (B) MEK inhibitor U0126 has no effect on tunicamycin- and thapsigargin-induced Akt phosphorylation. SMMC-7721 and Hep3B cells were treated with tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l) for indicated periods with or without U0126 (10 μmol/l) pre-incubation for 1 h. Whole-cell lysates were subjected to Western blot analysis of phosphorylated Akt and ERK. (C) Transient transfection of myr-HA-Akt inhibits ER stress-induced ERK phosphorylation. SMMC-7721 (upper panel) and Hep3B (bottom panel) cells were transient transfection with the control or myr-HA-Akt constructs. Twenty-four hours later, the cells were treated with tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l) for indicated periods. Whole-cell lysates were subjected to Western blot analysis of phosphorylated ERK. (D) PI3K inhibitor LY294002 inhibits tunicamycin- and thapsigargin-induced c-raf phosphorylation in SMMC-7721 cells. SMMC-7721 cells were treated with tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l) for 3 h with or without LY294002 (30 μmol/l) pre-incubation for 1 h. Whole-cell lysates were subjected to Western blot analysis of phosphorylated c-raf (Ser259) and Akt (Ser473).

rylation of c-raf on Ser259 under ER stress in SMMC-7721 cells (Fig. 4D), indicating that the PI3K/Akt pathway might inhibit MEK/ERK activation through phosphorylating c-raf on Ser259 under ER stress.

The cross-talk between PI3K/Akt and MEK/ERK mediates ER stress-induced cell cycle progress. We tested whether the cross-talk between the PI3K/Akt and MEK/ERK cascades was involved in ER stress-induced cell-cycle arrest. Firstly, the effects of PI3K/Akt inactivation on cell cycle distribution were studied in SMMC-7721 and HepG2 cells under ER stress. PI3K inhibitor LY294002 pre-incubation caused significant reduction in the fraction of G_0/G_1 phase cells, with a concomitant increases in the percentage of G_2/M phase cells in SMMC-7721 cells, compared with cells under ER stress conditions without LY294002 for the same period (Fig. 5). Furthermore, ER stress-induced cell cycle arrest was inhibited by myr-Akt overexpression (Fig. 5). Similar results were observed in HepG2 cells (data not shown). Then, the roles of MEK/ERK activation in ER stress-induced cell cycle progress were tested. It is notable that MEK inhibitor U0126 did not promote tunicamycin- or thapsigargin-induced cell cycle arrest but moderately increased cell percentage in the S phase in SMMC-7721 cells (Fig. 5), indicating MEK/ERK activation contributes to ER stress-induced cell cycle arrest. These data suggest that the cross-talk between the PI3K/Akt and MEK/ERK cascades is involved in ER stress-induced cell cycle arrest.

The cross-talk between PI3K/Akt and MEK/ERK mediates ER stress-induced cell death. It has been suggested that endogenous PI3K/Akt is involved in ER stress and plays a critical role in cell survival by suppressing ER stress-induced cell death (15). We tested whether the PI3K/Akt pathway is involved in protection of HCC cells against ER stress-induced apoptosis. The PI3K inhibitor LY294002 pre-incubation sensitized SMMC-7721 and HepG2 cells to tunicamycin- and thapsigargin-induced apoptosis ($P<0.05$) (Fig. 6). The PI3K inhibitor alone did not cause appreciable

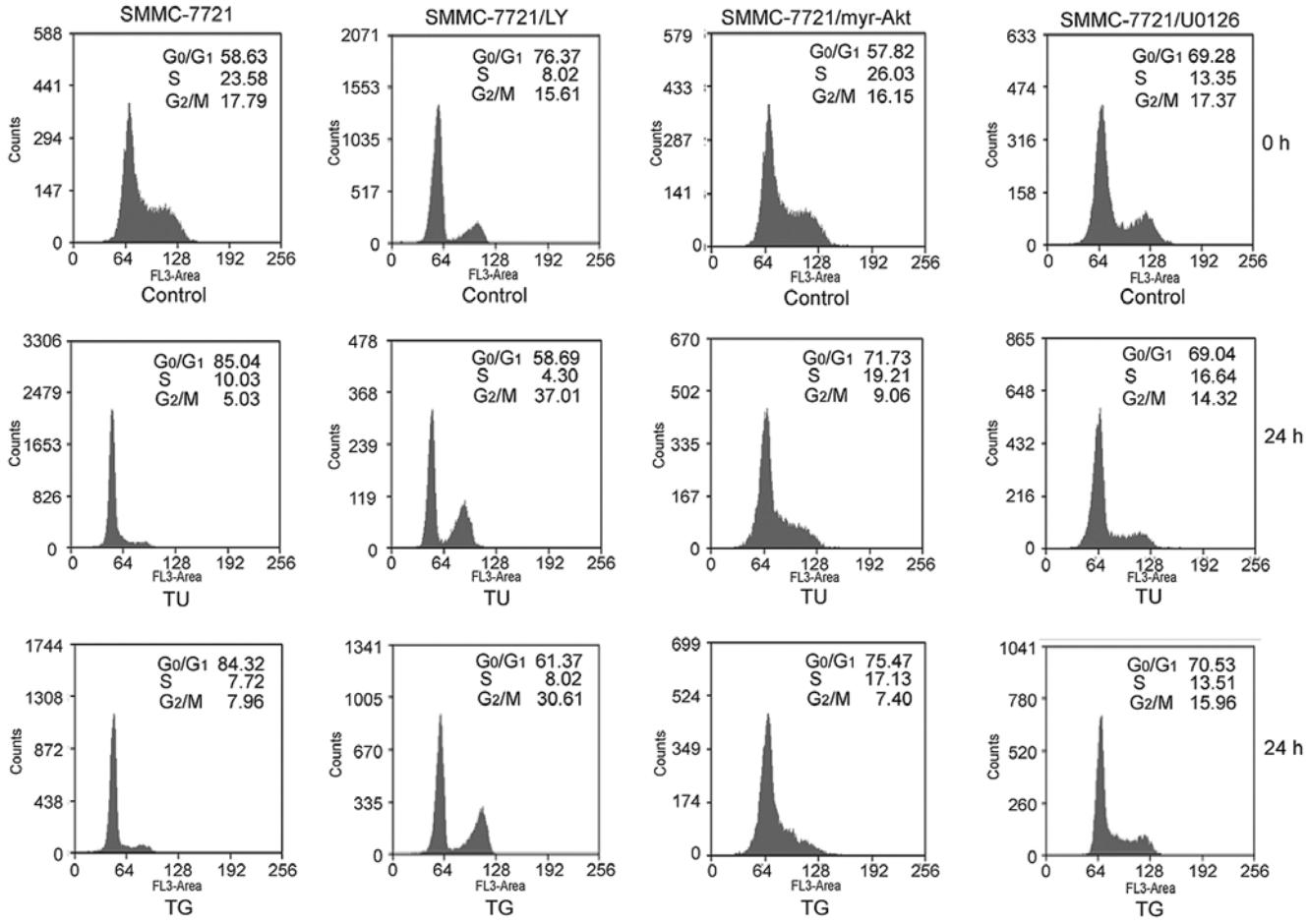

Figure 5. Cross-talk between the PI3K/Akt and MEK/ERK pathways mediates ER stress-induced HCC cell cycle arrest. PI3K inhibitor LY294002 increases cell percentage in the G_2/M phases and decreases cell percentage in the G_0/G_1 phases. Myr-Akt transient transfection or MEK inhibitor U0126 inhibits tunicamycin-induced cell cycle arrest. SMMC-7721 cells with tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l) treatment for 24 h with or without LY294002 (30 μmol/l) or U0126 (10 μmol/l) pre-incubation for 1 h were subjected to measurement of cell cycle distribution using flow cytometry after staining with propidium iodide. Myr-Akt expressed SMMC-7721 cells were treated with tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l) for 24 h, then subjected to measurement of cell cycle distribution using flow cytometry after staining with propidium iodide. Data are representative of three individual experiments.

apoptotic cell death in either cell line. As ER stress gradually induced endogenous MEK/ERK activation in HCC cells, we hypothesise that MEK/ERK might play a more critical role in counteracting ER stress-induced apoptosis than that of Akt. As the results showed, MEK inhibitor U0126 significantly sensitized SMMC-7721 and HepG2 cells to tunicamycin- and thapsigargin-induced apoptosis ($P<0.05$) (Fig. 6). The MEK inhibitor U0126 alone did not cause appreciable apoptotic cell death in either cell line. Thus, the inactivation of PI3K/Akt mediated MEK/ERK activation protects HCC cells from ER stress-induced apoptosis. It is important to note that MEK inhibitor U0126 rendered HCC cells more sensitive to ER stress-induced apoptosis than PI3K inhibitor LY294002 ($P<0.05$) (Fig. 6).

Discussion

Cells have developed a sophisticated cellular response, the unfolded protein response (UPR), to cope with ER stress. The PERK branch of the UPR protects the cell from protein misfolding overload in the ER by inducing a global protein translation repression (2,8,18). Translational attenuation confers immediate protective advantages, but could also be cytotoxic if the protein synthesis drop below levels necessary to sustain vital functions. A consequence of translation attenuation is the loss of cyclin D1 and a subsequent cell cycle arrest primarily in the G_1 phase (10). It is reasonable that ER stress-induced cell cycle arrest decreases the basal levels necessary to sustain vital functions and provides cells with much time to re-establish ER homeostasis. Therefore, ER stress-induced cell cycle arrest in HCC cells is an adaptive response for survival. In ER stress conditions, PERK-dependent cyclin D1 loss requires its phosphorylation on eIF2α (2), which in turn prevents formation of translation initiation complexes, thereby blocking protein synthesis. In the present study, we found that overexpression of cyclin D1 resulted in reduction in the fraction of G_1 phase cells, with a concomitant increase in the percentage of G_2 phase cells under ER stress in HCC cells, indicating that, in additional to PERK/eIF2α/cyclin D1 module, ER stress also activates other mechanisms, which attribute to cell cycle arrest in HCC cells. These mechanisms involve ER stress-induced cross-talk between the PI3K/Akt and MEK/ERK pathways.

The PI3K/Akt and MEK/ERK cascades are central regulators of cell metabolism, proliferation and survival (19-21). Cross-talk between the PI3K/Akt and Raf/MEK/ERK

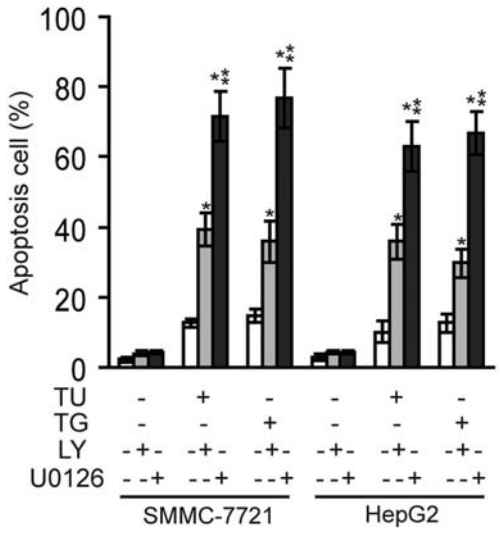

Figure 6. Cross-talk between PI3K/Akt and MEK/ERK pathways mediates ER stress-induced cell death. PI3K inhibitor LY294002 and MEK inhibitor U0126 sensitize HCC cells to ER stress-induced apoptosis. SMMC-7721 and HepG2 cells were treated with tunicamycin (5 μg/ml) or thapsigargin (1 μmol/l) for 48 h with or without LY294002 (30 μmol/l) or U0126 (10 μmol/l) pre-incubation for 1 h. Apoptosis was measured using flow cytometry after staining with FITC-conjugated Annexin V and propidium iodide. Columns, mean of three individual experiments; bars, SE. *Significantly different from control value; **significantly different from *value.

pathways occurs at multiple levels. Some studies indicate that PI3K activity is essential for the Raf/MEK/ERK cascade activation (22). Other studies suggest that the PI3K/Akt pathway synergizes with the Raf/MEK/ERK pathway to provide a more robust signal (16,23). In this study, we observed Akt and ERK were activated by ER stress with different kinetics in HCC cells. In SMMC-7721 and Hep3B cells, ER stress-induced Akt inactivation coordinated with ERK activation long-term, whereas insulin gave a modest duration of Akt and ERK activation simultaneously. More importantly, ER stress-induced ERK activation was clearly elevated when the PI3K/Akt pathway was blocked by either PI3K inhibitor LY294002 or overexpression of dominant-negative Akt. Furthermore, constitutively activated Akt mutant construct HA-myr-Akt markedly inhibited ERK phosphorylation under ER stress. In contrast, MEK inhibitor U0126 had no effect on Akt activation. We therefore speculate that, in SMMC-7721 and Hep3B cells, PI3K is an upstream signaling pathway mediating the activation of ERK under ER stress. A previous study reported that PI3K mediates ER stress-induced activation of ERK in the MCF-7 breast carcinoma cell line (15). The reason for the different effects of PI3K/Akt on ER stress-induced MEK/ERK activation remains unclear.

How is the MEK/ERK pathway inhibited by the PI3K/Akt pathway under ER stress in HCC cells? It has been reported that Akt can negatively regulate the Raf/MEK/ERK pathway through specifically phosphorylating Raf, such as on Ser259, in certain cell types (16,17,24). In this study, we found that LY294002 inhibited the phosphorylation of c-raf on Ser259 under ER stress in HCC cells, indicating that the PI3K/Akt pathway inhibits the MEK/ERK pathway, at least partly, through regulating the activity of c-raf under ER stress.

The PI3K/Akt pathway is negatively regulated in part by lipid phosphatase such as PTEN and SHIP (25-29). Akt also appears to be directly dephosphorylated by the Ser-Thr phosphatase PP2A in response to certain stimuli (30,31). We found that ER stress-induced Akt inactivation did not depend on PTEN or PP2A signal, but required C/EBP homologous protein (CHOP) induction (our unpublished results).

The PI3K/Akt and MEK/ERK pathways have been implicated in the regulation of both G_1/S cell cycle progression and G_2/M transition (32-38). Akt-mediated MEK/ERK cascade inactivation through phosphorylation of Raf may switch the biological response from growth arrest to proliferation in MCF-7 breast cancer cell line (39,40). Therefore, cross-talk between the PI3K/Akt and the MEK/ERK pathways in HCC cells under ER stress appears to be involved in cell cycle regulation. Here, we found that inhibition of the PI3K/Akt cascade caused significant increases in cell percentage in the G_2/M phase and decreases cell percentage in the G_0/G_1 phase. In addition, ER stress-induced cell cycle arrest was inhibited by enforced Akt activation. These results indicate that the down-regulation of Akt activation plays a role in cell cycle arrest under ER stress. Furthermore, we reasoned whether Akt inactivation-mediated ERK activation is required for cell cycle arrest under ER stress. Our data showed that MEK/ERK inhibition moderately inhibited ER stress-induced cell cycle arrest in HCC cells, suggesting that ERK activation promotes ER stress-induced cell cycle arrest. According to these results, we propose that the ER stress-induced cross-talk between the PI3K/Akt and MEK/ERK cascades acts as additional mechanisms in cell cycle regulation.

The PI3K/Akt and MEK/ERK pathways are central regulators of cell survival. Considering that long-term exposure to ER stress resulted in Akt inactivation, which was accompanied by increasing in ERK activation, it seems that the role of endogenous Akt activation in protecting HCC cells from apoptosis is not as important as endogenous MEK/ERK activation. In this study, we found that MEK inhibitor U0126 made HCC cells much more sensitive to ER stress-induced cell death than PI3K inhibitor LY294002. Thus, we propose that the cross-talk between the PI3K/Akt and MEK/ERK pathways under ER stress not only mediates cell cycle arrest, which is mainly dependent on decreased Akt activation, but also protects HCC cells from apoptosis, which is mainly dependent on increased ERK activation.

In summary, we show in this study that ER stress causes decreased Akt activation, which is correlated with increased ERK activation in HCC cells. The down-regulation of Akt activation mediates the up-regulation of ERK activation under ER stress. We also provided evidence that the cross-talk between the PI3K/Akt and MEK/ERK cascades contributes to both HCC cell cycle arrest and cell survival under ER stress. Thus, it is suggested that ER stress-induced cross-talk between the PI3K/Akt and MEK/ERK cascades acts as an adaptive response for HCC cells to cope with stress.

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HA-Akt), domain-negative HA-Akt (K179M) expression constructs and cyclin D1 expression construct, respectively.

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