Original Article

PI3K/Akt Promotes GRP78 Accumulation and Inhibits Endoplasmic Reticulum Stress-Induced Apoptosis in HEK293 Cells

(ER stress / PI3K/Akt / GRP78 / apoptosis / HEK293 cells)

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Abstract. The potential pro-survival role of phosphatidylinositol 3-kinase (PI3K)/Akt during endoplasmic reticulum stress has been well-characterized. However, the detailed mechanisms remain largely unknown. Here, we showed that PI3K/Akt inhibition promoted endoplasmic reticulum stress-induced apoptosis in a glucose-regulated protein 78 (GRP78)-dependent manner. During endoplasmic reticulum stress, high levels of Akt phosphorylation were sustained for at least 18 h in HEK293 cells. Importantly, PI3K/Akt enhanced GRP78 accumulation through increasing its stability following endoplasmic reticulum stress. Furthermore, Akt1, but not Akt2 or Akt3, was involved in GRP78 stability regulation. These results suggest that PI3K/Akt inhibits endoplasmic reticulum stress-induced apoptosis in HEK293 cells, at least in part, by promoting GRP78 protein stability.

Abbreviations: Akt – serine/threonine protein kinase, protein kinase B, ATF4/6 – activating transcription factor 4/6, CHX – cycloheximide, ER – endoplasmic reticulum, GADD153 – growth arrest and DNA damage-inducible protein 153, GRP78 – glucose-regulated protein 78, GRP94 – glucose-regulated protein 94, GSK3β – glycogen synthase kinase 3 β, HEK – human embryonic kidney, IRE1 – inositol-requiring protein 1, MDM2 – murine double minute-2, mTOR – mammalian target of rapamycin, PDK1/2 – phosphoinositide-dependent kinase 1/2, PERK – PRK (RNA-dependent protein kinase)-like ER kinase, PI3K– phosphatidylinositol 3-kinase, PKB – protein kinase B (Akt), PVDF – polyvinylidene fluoride, RT-PCR – reverse transcription-polymerase chain reaction, UPR – unfolded protein response, XBP1 – X-box binding protein 1.

Introduction

The endoplasmic reticulum (ER) is the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins. When the function of ER is impaired by disturbances (such as disruption of Ca^{2+} homeostasis, inhibition of protein glycosylation or disulfide bond formation and hypoxia), the unfolded or misfolded proteins accumulate in the ER lumen, which subsequently triggers an evolutionarily conserved response, termed the unfolded protein response (UPR) (Mori, 2000; Harding et al., 2002; Rutkowski and Kaufman, 2004). The activation of UPR is believed to alleviate ER stress and promote cell survival (Gething and Sambrook, 1992; Harding et al., 1999; Mori, 2000; Harding et al., 2002; Rutkowski and Kaufman, 2004). Glucose-regulated protein 78 (GRP78), an UPR target gene, is one of the best-characterized ER chaperone proteins. Under unstressed conditions, the luminal domains of ER stress sensors, inositol-requiring protein 1 (IRE1), activating transcription factor 6 (ATF6), and PRK (RNA-dependent protein kinase)-like ER kinase (PERK), are occupied by GRP78, which represses the UPR signalling pathways. Upon ER stress, sequestration of GRP78 by unfolded proteins activates these sensors, inducing UPR (Hendershot, 2004; Schröder and Kaufman, 2005). GRP78 induction plays pivotal roles in maintaining ER homeostasis and protecting the cells against ER stress-induced apoptosis (Hendershot, 2004; Lee, 2005; Dong et al., 2008).

Akt/protein kinase B (PKB) has been identified as a direct target of phosphatidylinositol 3-kinase (PI3K) (Franke et al., 1995; Datta et al., 1996). Akt is phosphorylated at Thr308 and Ser473 by 3-phosphoinositide-dependent kinase 1/2 (PDK1/2) and is fully activated when both residues are phosphorylated (Anderson et al., 1998; Stephens et al., 1998). PI3K/Akt is a critical mediator of growth factor-induced cell survival and has been shown to suppress cell death induced by a variety of apoptotic

stimuli (Marte and Downward, 1997). It has been reported that Akt activation was increased by short-term exposure to ER stress but was down-regulated by long-term exposure to ER stress, and Akt inactivation plays an important role in growth arrest and DNA damage-inducible protein 153 (GADD153) induction (Hyoda et al., 2006; Hosoi et al., 2007). Although PI3K/Akt has been shown to prevent ER stress-induced cell death (Hu et al., 2004; Hyoda et al., 2006), the detailed mechanisms remain largely unknown. Here, we found that the PI3K/Akt pathway is critical for ER stress-induced GRP78 accumulation, which subsequently facilitates the cells to cope with ER stress.

Material and Methods

Material

Thapsigargin (TG) and dithiothreitol (DTT) were purchased from Sigma Chemical Company (St. Louis, MO). PI3K inhibitor LY294002, mitogen-activated protein kinase (MAPK) or Erk kinase (MEK)1/2 inhibitor U0126, mammalian target of rapamycin (mTOR) inhibitor rapamycin and glycogen synthase kinase (GSK)3β inhibitor TDZD-8 were purchased from Merck Chemicals (Darmstadt, Germany). Antibodies against GRP78, GRP94, cleaved poly-(ADP-ribose) polymerase (PARP), GADD153, phospho-Akt1 and Akt1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against HA-tag, Myc-tag, phospho-Akt (Ser473), Akt, phospho-mTOR (Ser2448), mTOR, phospho-p70 S6 kinase (Thr389), p70 S6 kinase, phospho-extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204), ERK1/2 and β-actin were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture and treatment

Human embryonic kidney (HEK) 293 cells and mouse embryonic fibroblast cell line (NIH 3T3) cells were maintained at 37 °C in a humidified incubator containing 5 % CO_2, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % foetal bovine serum and 1 % penicillin/streptomycin. Dithiothreitol (2.5 mM) and thapsigargin (1 μM) were used to induce ER stress response. The cells were pretreated with 30 μM LY294002, 10 μM U0126 or 10 μM TDZD-8 for 1 h prior to dithiothreitol or thapsigargin treatment to block PI3K/Akt, MEK/ERK or GSK3β. The constitutively active expression vectors for Akt (myr-HA-Akts) and the dominant negative kinase-dead mutant vector for HA-Akt1 (K179M) were kindly provided by Professor Jin Q. Cheng. Transient transfection of HA-Akts, GRP78, murine double minute-2 (MDM2), and the control constructs were performed using polyethylenimine (PEI) (Invitrogen, Carlsbad, CA). The oligonucleotides and protocol used for Akt isoforms and GRP78 knockdown have been previously described (Li and Lee, 2006; Hara et al., 2008); the control siRNA against GFP was used.

Reverse transcription PCR and real-time PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The reverse transcription reactions were carried out using the M-MLV Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's protocol. The PCRs were optimized for a number of cycles to ensure product intensity within the linear phase of amplification. PCR products were separated by electrophoresis in 2% or 4% agarose gel, stained with ethidium bromide. All tests were repeated three times, and one of the repeats was shown in the results. Real-time PCR analyses were performed using SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan). Results were normalized with 18S. The primers used in this study are as follows: human GRP78 sense primer, 5'-ATC ACG CCG TCC TAT GTC GC-3' and anti-sense primer, 5'-TCT CCC CCT CCC TCT TAT CC-3'; human XBP1 sense primer, 5'-CCT TGT AGT TGA GAA CCA GG-3' and anti-sense primer, 5'-GGG GCT TGG TAT ATA TGT GG-3'; human 18S sense primer, 5'-GGG AGG TAG TGA CGA AAA AT-3' and anti-sense primer, 5'-ACC AAC AAA ATA GAA CCG CG-3'.

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 g for 15 min. Protein concentrations were measured using the BCA assay (Santa Cruz, CA). Equal 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 the relevant primary antibodies, washed three times with TBST (TBS containing 0.1 % Tween-20), and probed with the appropriate horseradish-peroxidase-conjugated secondary antibodies at room temperature for 2 h. Immunoreactive material was detected using the ECL kit (Santa Cruz, CA) according to the manufacturer's instruction. The bands were quantified densitometrically.

Apoptosis analysis

Cells were treated with dithiothreitol (2.5 mM) and thapsigargin (1 μM) for the indicated time. Apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit (BD PharMingen, Franklin Lakes, NJ) according to the manufacturer's manual. Annexin V staining was analysed by flow cytometry within 1 h. The experiments were repeated three times.

Statistical analysis

Results are expressed as mean ± standard deviation. Statistical analysis was performed using Student's t-test. $P < 0.05$ was considered statistically significant.

Results

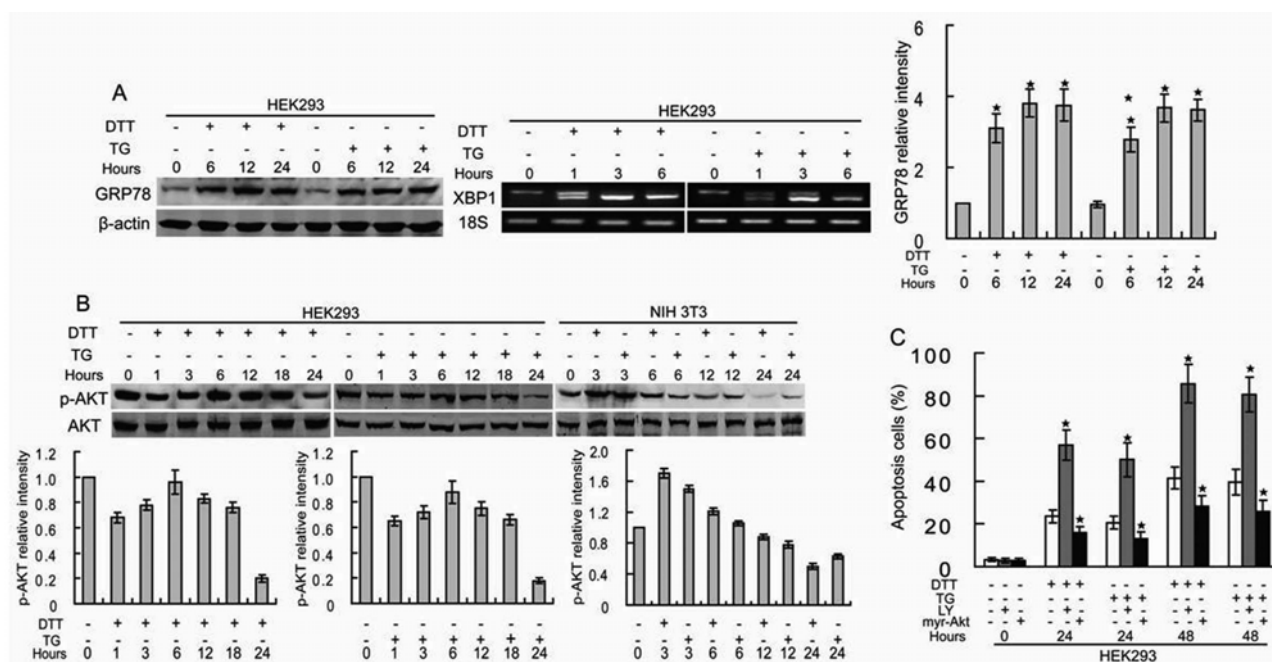### PI3K/Akt is involved in ER stress-induced apoptosis in HEK293 cells

Incubation of HEK293 cells with ER stress inducers dithiothreitol and thapsigargin markedly elevated GRP78 protein level and rapidly induced XBP1 mRNA splicing (Fig. 1A), indicating UPR activation (Yoshida et al., 2001a; Hu et al., 2004). It has been reported that long-term exposure to ER stress stimuli results in rapidly decreasing PI3K/Akt activity (Hu et al., 2004; Hyoda et al., 2006; Hosoi et al., 2007). During ER stress, the phosphorylation levels of Akt in HEK293 cells were investigated. Western blot analysis showed that high levels of Akt phosphorylation were sustained for at least 18 h after dithiothreitol and thapsigargin treatment (Fig. 1B). It is notable that phospho-Akt was slightly reduced within 3 h following ER stress, and restored in 6 h. An obvious decrease in the phosphorylation level of Akt was observed 24 h after dithiothreitol and thapsigargin treatment (Fig. 1B). Consistent with previous data (Hyoda et al., 2006), we found that Akt protein levels were not changed in response to ER stress (Fig. 1B). Furthermore, Fig. 1B shows that Akt activation was increased within 6 h under ER stress but was decreased by long-term exposure to ER stress stimuli. Taken together, these data indicate that PI3K/Akt plays an important role in HEK293 cells under ER stress.

Next, we examined whether the PI3K/Akt pathway can protect HEK293 cells against ER stress-induced death. HEK293 cells were treated with dithiothreitol and thapsigargin in the presence or absence of PI3K-specific inhibitor LY294002 (30 μM). The results showed that LY294002 significantly sensitized HEK293 cells to dithiothreitol- and thapsigargin-induced apoptosis (Fig. 1C). Furthermore, myr-HA-Akt transient transfection inhibited ER stress-induced apoptosis in HEK293 cells (Fig. 1C). These results reveal that the activity of PI3K/Akt is critical for protecting HEK293 cells from the ER stress-induced apoptosis.
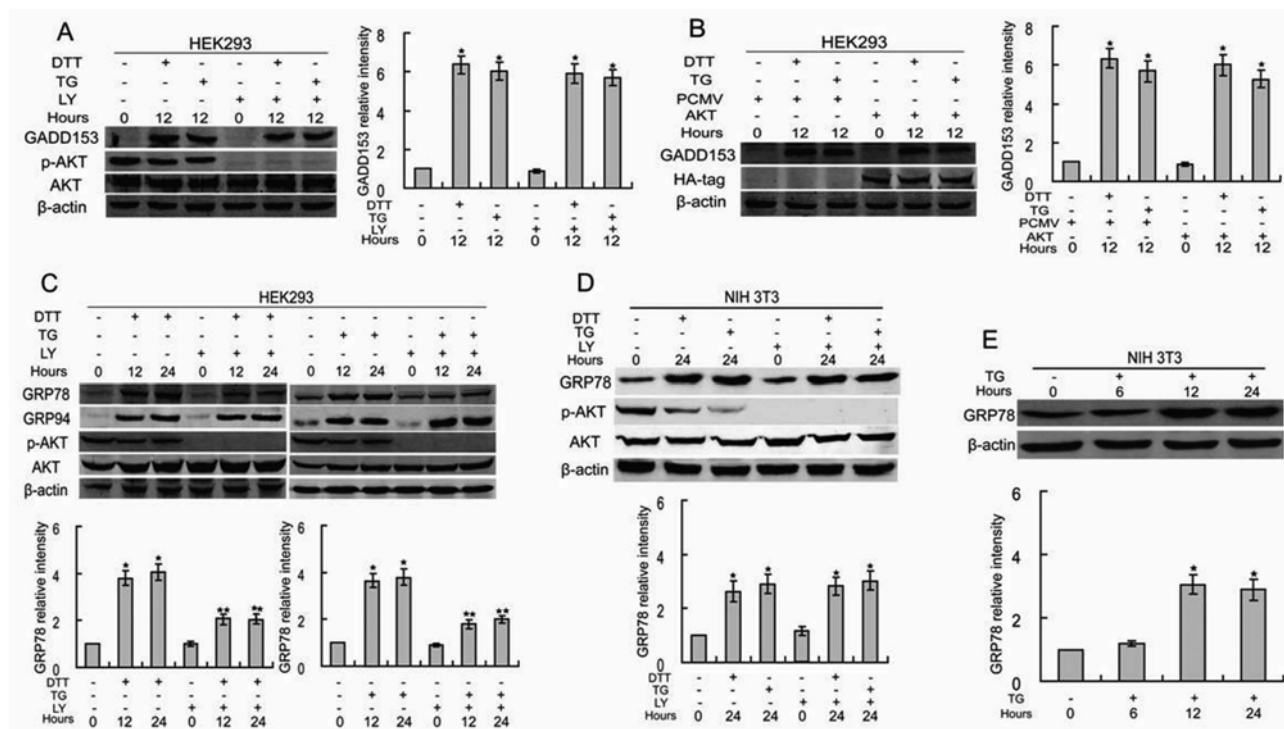
PI3K/Akt is required for GRP78 accumulation in HEK293 cells

It has been reported that PI3K/Akt can inhibit ER stress-mediated GADD153, an important promoter of apoptosis induction (Hyoda et al., 2006; Hosoi et al., 2007). We investigated whether PI3K/Akt plays some role in regulating ER stress-mediated GADD153 induction in HEK293 cells. As shown in Figs. 2A and 2B, PI3K/Akt inhibition or myr-HA-Akt transient transfection had no appreciable effect on GADD153 induction in dithiothreitol- and thapsigargin-treated HEK293 cells. These data imply that PI3K/Akt is not involved in GADD153 induction in HEK293 cells under ER stress.

Since the sustained high levels of Akt phosphorylation were observed in ER-stressed HEK293 cells, we suspected that PI3K/Akt might play some roles in GRP78

Fig. 1. PI3K/Akt is involved in ER stress-induced HEK293 cell apoptosis. (A) Dithiothreitol (2.5 mM)- and thapsigargin (1 μM)-induced UPR in HEK293 cells. GRP78 protein, unspliced and spliced XBP-1 mRNA were detected by Western blot and RT-PCR, respectively. (B) Western blot analysis for the phosphorylation of Akt in HEK293 and NIH 3T3 cells after dithiothreitol (2.5 mM) or thapsigargin (1 μM) treatment for indicated times. (C) HEK293 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM) for 24 or 48 h with or without LY294002 (30 μM) pre-incubation for 1 h or myr-Akt transient transfection. 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.

Fig. 2. PI3K/Akt is required for GRP78 accumulation in HEK293 cells. (A) LY294002 (30 μM) pretreatment for 1 h had no effect on GADD153 protein induction in dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-treated HEK293 cells. (B) Akt transient transfection had no effect on GADD153 protein induction in dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-treated HEK293 cells. (C) LY294002 (30 μM) pretreatment for 1 h inhibited dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-induced GRP78, but not GRP94, protein accumulation in HEK293 cells. (D) LY294002 (30 μM) pretreatment for 1 h had no effect on ER stress-induced GRP78 expression in NIH 3T3 cells. (E) Thapsigargin (1 μM) treatment induced GRP78 expression in NIH 3T3 cells.

accumulation. This hypothesis was supported by our data, which demonstrated that PI3K inhibitor LY294002 (30 μM) pre-incubation inhibited GRP78 protein up-regulation in dithiothreitol- and thapsigargin-treated HEK293 cells at the indicated time (Fig. 2C). Moreover, Fig. 2C shows that ER stress-induced GRP94, another ER chaperone, expression was not affected by PI3K/Akt inhibition, which suggests that the regulation of GRP78 by PI3K/Akt under ER stress is specific. The role of PI3K/Akt in GRP78 regulation was also investigated in NIH 3T3 cells, and the data showed that LY294002 (30 μM) pre-incubation had no effect on ER stress-mediated GRP78 induction (Fig. 2D). Furthermore, Akt1, but not Akt2 or Akt3, transient transfection obviously up-regulated the protein level of GRP78 in dithiothreitol- and thapsigargin-treated HEK293 cells (Fig. 3A). These results indicate that Akt1 is the predominant isoform that is involved in regulating GRP78 accumulation.

To further confirm the role of Akt1 in GRP78 accumulation, HEK293 cells were transiently transfected with the dominant-negative Akt1 (K179M) or the control constructs 24 h before dithiothreitol and thapsigargin treatment. It is notable that Akt1 (K179M) not only blocked the phosphorylation of Akt1, but also inhibited ER stress-induced GRP78 protein accumulation (Fig. 3B). ER stress stimuli induced no apparent changes in the level of phospho-Akt1; this may be due to the fact that differ-

ent Akt isoforms have different dynamic features of kinase activity following ER stress. Consistent with these data, Akt1 (but not Akt2 or Akt3) siRNA significantly down-regulated GRP78 protein level in dithiothreitol- and thapsigargin-treated HEK293 cells (Fig. 3C). Thus, it is confirmed that Akt1, but not Akt2 or Akt3, is involved in GRP78 accumulation.

Exogenous GRP78 expression inhibits PI3K/Akt inhibition-mediated apoptosis in HEK293 cells

Considering that PI3K/Akt inactivation decreases GRP78 protein accumulation, it seems that GRP78 may contribute to the anti-apoptotic function of PI3K/Akt. To confirm the role of GRP78 in preventing HEK293 cells from ER stress-induced apoptosis, GRP78 expression was suppressed by the GRP78-specific siRNA. Fig. 4A shows that GRP78 suppression substantially increased the sensitivity of HEK293 cells to dithiothreitol- and thapsigargin-induced apoptosis. Moreover, GRP78 overexpression inhibited dithiothreitol- and thapsigargin-mediated HEK293 cell apoptosis (Fig. 4A). In order to make sure whether exogenous GRP78 expression inhibits PI3K/Akt inactivation-mediated cell death under ER stress, HEK293 cells with or without GRP78 vector transient transfection were treated with LY294002 (30 μM) before dithiothreitol and thapsigargin administration. As shown in Fig. 4B, GRP78 over-expression inhibited
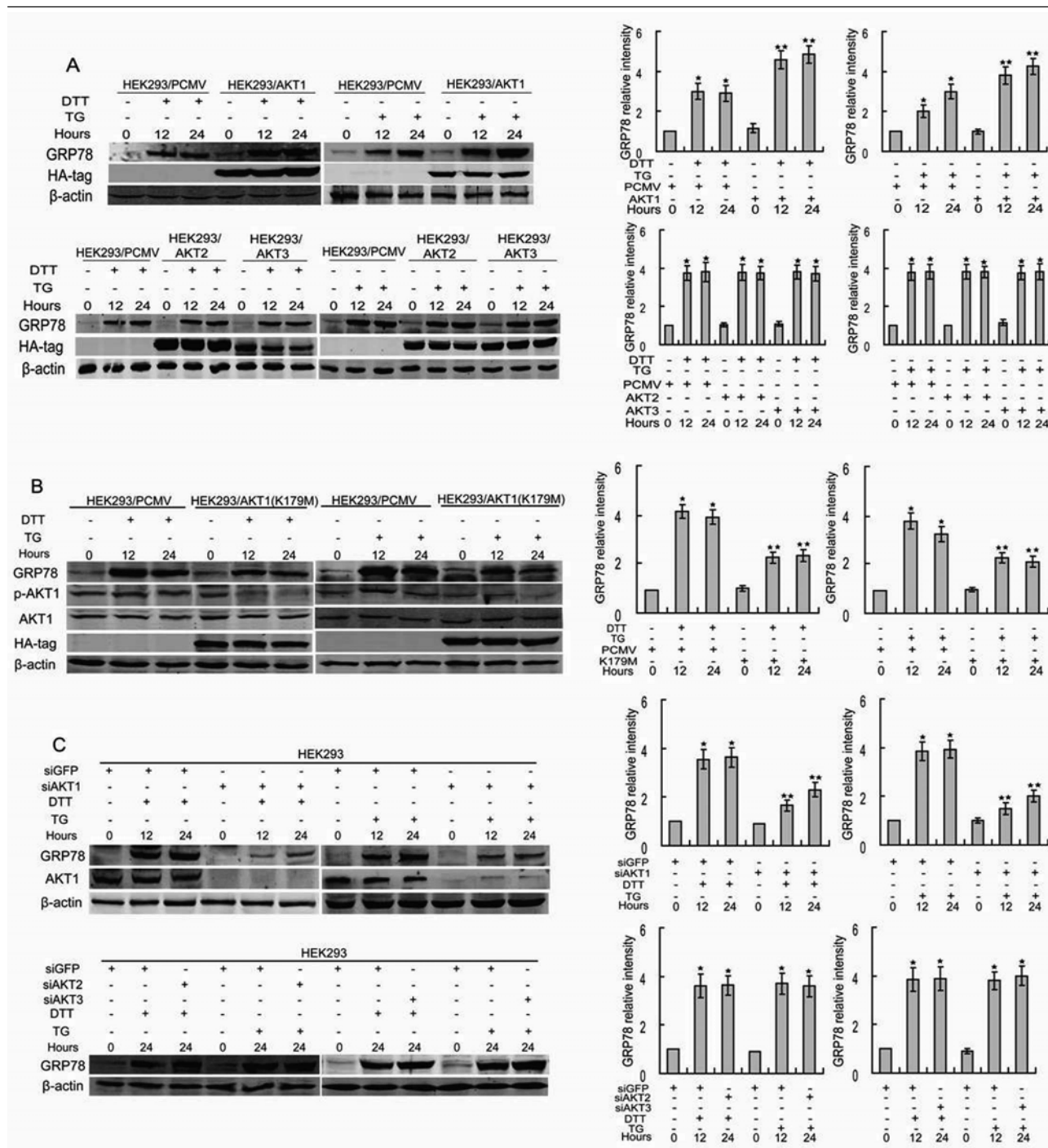
Fig. 3. Akt1 is the predominant isoform that is involved in regulating GRP78 accumulation. (A) Akt1 transient transfection up-regulated the GRP78 protein level in dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-treated HEK293 cells, whereas transfection with Akt2 or Akt3 had no effect. (B) Domain-negative Akt1 transient transfection suppressed ER stress-induced GRP78 protein expression in HEK293 cells. (C) Akt1 siRNA transient transfection suppressed ER stress-induced GRP78 protein expression in HEK293 cells, whereas transfection with Akt2 or Akt3 siRNA had no effect.

PI3K/Akt inhibition-mediated apoptosis in dithiothreitol- or thapsigargin-treated HEK293 cells. Furthermore, GRP78 over-expression inhibited Akt1 (K179M)- and siAkt1-induced apoptosis under ER stress (Fig. 4C). These observations suggest that the cytoprotective profile of PI3K/Akt under ER stress is dependent, at least in part, on GRP78 accumulation. Furthermore, GRP78 suppression did not completely block the protective role of PI3K/Akt in dithiothreitol- and thapsigargin-treated

HEK293 cells (Fig. 4D), indicating that GRP78 suppression reduced the cytoprotective ability of PI3K/Akt under ER stress only in part.

PI3K/Akt regulates GRP78 expression at the protein level in HEK293 cells

We next studied whether PI3K/Akt regulates GRP78 induction at the transcription level. After HEK293 cells were treated with dithiothreitol and thapsigargin for in-
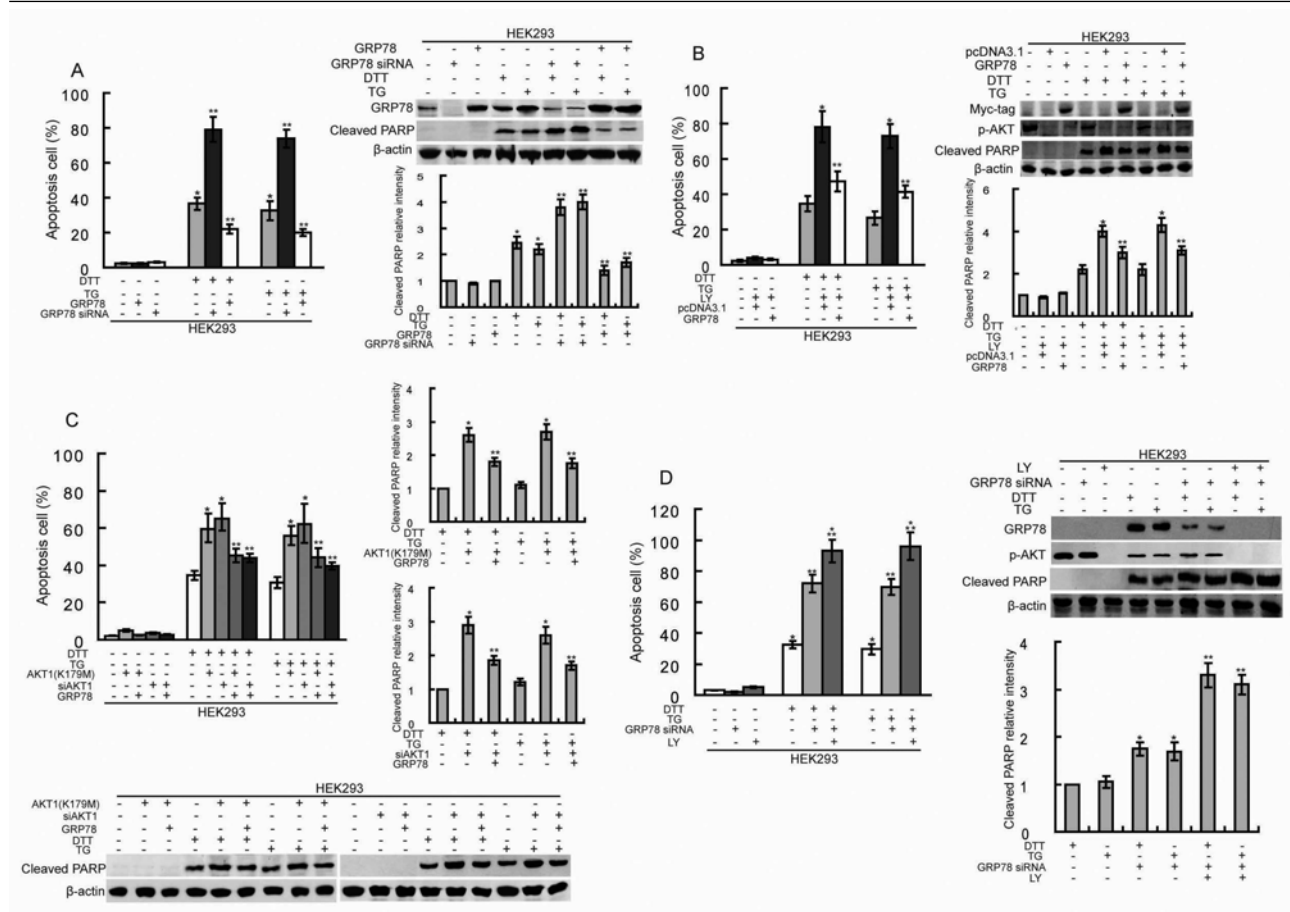
Fig. 4. GRP78 over-expression protects HEK293 cells against PI3K/Akt inhibition-mediated apoptosis. (A) GRP78 protects HEK293 cells from ER stress-induced apoptosis. After transfection with GRP78 construct or siRNA for 24 h, HEK293 cells were treated with or without dithiothreitol (2.5 mM) and thapsigargin (1 μM) for another 36 h. Apoptosis was evaluated by AnnexinV-FITC flow cytometry and reconfirmed by PARP cleavage. Columns, mean of three individual experiments; bars, SE. *Significantly different from control value; **significantly different from *value. (B) After transient transfection with the control or GRP78 construct for 24 h, HEK293 cells were treated with or without dithiothreitol (2.5 mM) and thapsigargin (1 μM) in the presence or absence of LY294002 (30 μM) for another 36 h. Apoptosis was evaluated by AnnexinV-FITC flow cytometry and reconfirmed by PARP cleavage. Columns, mean of three individual experiments; bars, SE. *Significantly different from control value; **significantly different from *value. (C) GRP78 over-expression protects HEK293 cells from Akt1 inhibition-induced apoptosis. After transient transfection with GRP78 construct, Akt1 (K179M) construct or Akt1 siRNA for 24 h, HEK293 cells were treated with or without dithiothreitol (2.5 mM) and thapsigargin (1 μM) for another 36 h. Apoptosis was evaluated by AnnexinV-FITC flow cytometry and reconfirmed by PARP cleavage. Columns, mean of three individual experiments; bars, SE. *Significantly different from control value; **significantly different from * value. (D) PI3K/Akt inhibition aggravates GRP78 suppression-enhanced HEK293 cell apoptosis. After transfection with GRP78 siRNA for 24 h, HEK293 cells were treated with dithiothreitol (2.5 mM) and thapsigargin (1 μM) in the presence or absence of LY294002 (30 μM) for another 36 h. Apoptosis was evaluated by AnnexinV-FITC flow cytometry and reconfirmed by PARP cleavage. Columns, mean of three individual experiments; bars, SE. *Significantly different from control value; **significantly different from *value.

dicated time periods in the presence or absence of 30 μM LY294002, real-time PCR analysis was performed to detect the effect of PI3K/Akt inhibition on GRP78 transcription. As shown in Fig. 5A, PI3K/Akt inhibition did not suppress ER stress-induced GRP78 mRNA expression in HEK293 cells. Furthermore, Akt1 over-expression did not cause GRP78 mRNA elevation (Fig. 5B). Akt2 and Akt3 over-expression also had no appreciable effects on the GRP78 mRNA induction (data not shown). These data indicate that PI3K/Akt does not participate in GRP78 mRNA induction following ER stress.

Thus, it is reasonable that PI3K/Akt regulates ER stress-induced GRP78 accumulation at the protein level.

It has been suggested that mTOR, an established target of Akt, is involved in cell survival and proliferation through regulating protein synthesis (Chung et al., 2002; Wendel et al., 2004). Therefore, it is interesting to investigate whether PI3K/Akt cascade mediates GRP78 protein induction through mTOR. We found that the phosphorylation of mTOR at Ser2448 was blocked by LY294002 (30 μM) in dithiothreitol- and thapsigargin-treated HEK293 cells (Fig. 5C). Further data showed
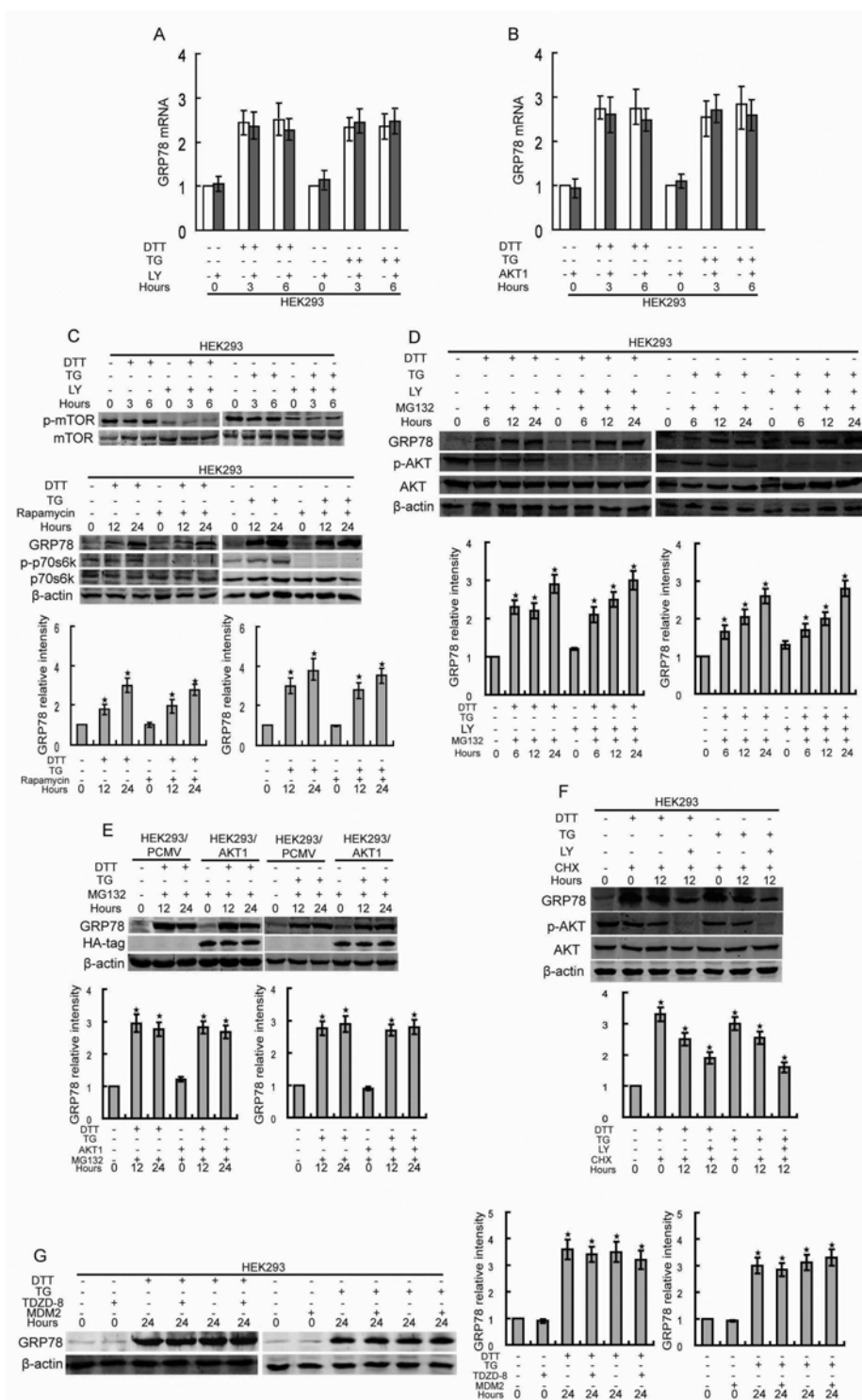
Fig. 5. PI3K/Akt mediates GRP78 accumulation at the protein level. (A, B) LY294002 (30 μM) and Akt1 had no effects on the GRP78 mRNA induction in dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-treated HEK293 cells. GRP78 mRNA levels were analysed by real-time RT-PCR. Columns, mean of three individual experiments; bars, SE. (C) LY294002 (30 μM) treatment inhibited the mTOR phosphorylation level (top panels), and mTOR inhibitor rapamycin had no effect on GRP78 protein expression (bottom panels) in dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-treated HEK293 cells at the indicated times. (D) LY294002 (30 μM)-mediated GRP78 down-regulation in dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-treated HEK293 cells was blocked by MG-132 (20 μM) administration for up to 6 h before cell harvest. (E) Akt1-mediated GRP78 up-regulation in dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-treated HEK293 cells was blocked by MG-132 (20 μM) administration for up to 6 h before cell harvest. (F) CHX had no effect on LY294002-mediated GRP78 down-regulation. After treatment with dithiothreitol (2.5 mM) or thapsigargin (1 μM) for 12 h, HEK293 cells were treated with CHX (50 μg/ml) for another 12 h in the presence or absence of 30 μM LY294002. (G) GSK3β inhibitor TDZD-8 and MDM2 construct had no effect on GRP78 protein induction in HEK293 cells.
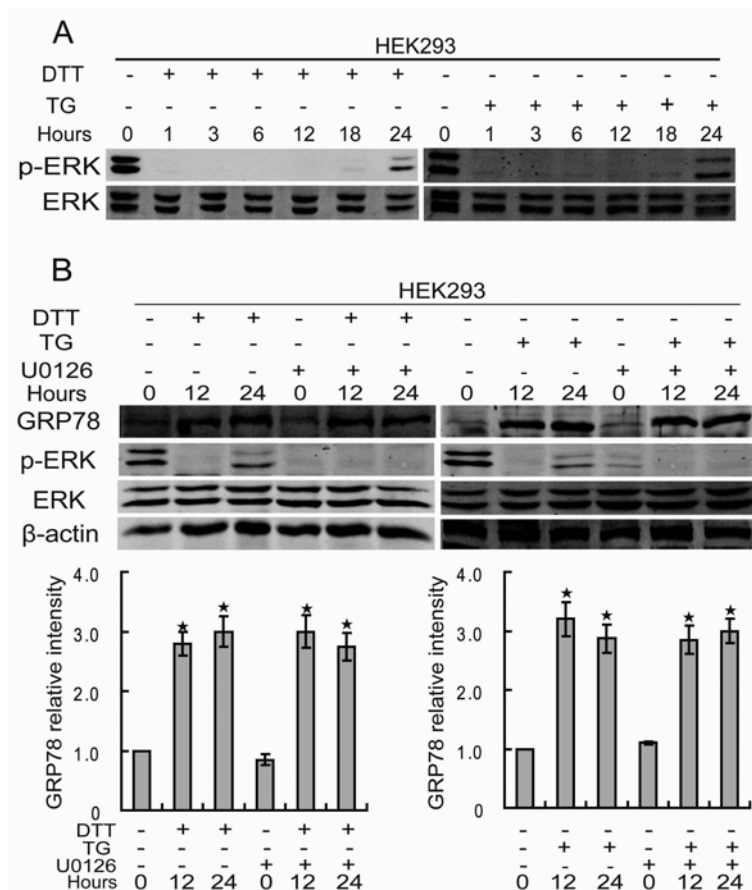
Fig.6. MEK/ERK is not involved in GRP78 induction in HEK293 cells. (A) Western blot analysis for the phosphorylation of ERK in HEK293 cells after dithiothreitol (2.5 mM) or thapsigargin (1 μM) treatment at indicated times. (B) U0126 (10 μM) pretreatment for 1 h had no effect on GRP78 protein induction in dithiothreitol (2.5 mM)- or thapsigargin (1 μM)-treated HEK293 cells.

that pretreatment with 50 nM mTOR inhibitor rapamycin for 1 h had no effect on ER stress-induced GRP78 protein expression (Fig. 5C). These data suggest that ER stress induces activation of mTOR in an Akt-dependent manner; however, this process is not involved in GRP78 accumulation.

Under normal conditions, the endogenous GRP78 protein is very low in HEK293 cells (Fig. 2C, Fig. 3A), despite the presence of relatively high basal levels of GRP78 transcripts, indicating its poor stability in HEK293 cells. Our data showed that proteasome inhibitor MG-132 (20 μM) treatment inhibited LY294002-mediated GRP78 protein reduction in HEK293 cells under ER stress (Fig. 5D), suggesting that PI3K/Akt plays a vital role in controlling the stability of GRP78 protein. Furthermore, MG-132 (20 μM) treatment inhibited Akt1 over-expression-mediated GRP78 protein increase (Fig. 5E). To test whether PI3K/Akt promotes GRP78 protein synthesis, HEK293 cells were treated with protein synthesis inhibitor cycloheximide (CHX) with or without LY294002 after dithiothreitol or thapsigargin treatment for 12 h. As shown in Fig. 5F, CHX has no effect on PI3K/Akt inhibition-mediated GRP78 protein reduction. Taken together, these data confirm the idea that PI3K/Akt promotes GRP78 accumulation

through increasing the stability of GRP78 protein. As GSK3β and MDM2, which both play pivotal roles in the regulation of protein stability, are established targets of Akt (Cross et al., 1995; Mayo and Donner, 2001), we also investigated whether GSK3β and MDM2 are involved in Akt-mediated GRP78 up-regulation. Fig. 5G shows that neither GSK3β inhibition nor MDM2 over-expression had effect on GRP78 induction, suggesting that the effect of Akt on GRP78 regulation is not mediated by GSK3β and MDM2.

MEK/ERK is not required for GRP78 induction in HEK293 cells

Previous studies have shown that ERK1/2 is constitutively activated in various cell types under ER stress, and the MEK/ERK pathway is believed to play an important role in ER stress-induced GRP78 induction (Arai et al., 2004; Jiang et al., 2007; Zhang et al., 2009). However, we found that the phosphorylation of ERK1/2 rapidly decreased (within 1 h) in dithiothreitol- or thapsigargin-treated HEK293 cells (Fig. 6A). Re-phosphorylation of ERK1/2 was observed at 24 h (Fig. 6A). These data imply that MEK/ERK might play little role in GRP78 induction in HEK293 cells. To test this hypothesis, we examined whether GRP78 induction could be

blocked by MEK/ERK inhibition. We monitored protein levels of GRP78 following exposure of HEK293 cells to dithiothreitol or thapsigargin with or without 10 μM U0126 pretreatment. As shown in Fig. 6B, MEK/ERK inhibition had no effect on ER stress-induced GRP78 expression, indicating that the induction of GRP78 is independent of MEK/ERK activity in HEK293 cells.

Discussion

It has been demonstrated that PI3K/Akt plays a pivotal role in regulating cell survival under ER stress conditions (Hu et al., 2004; Srivanasan et al., 2005), but the underlying mechanisms remain unclear. In this study, we reported that the PI3K/Akt pathway protects HEK293 cells against ER stress-induced apoptosis through GRP78 regulation. During ER stress, GRP78 plays an essential role in protection of cells from apoptosis (Hendershot, 2004; Dong et al., 2008). Here, we found that PIK/Akt was required for ER stress-induced GRP78 accumulation in HEK293 cells. Furthermore, the findings that knockdown of GRP78 by siRNA enhanced apoptosis induced by dithiothreitol or thapsigargin, and GRP78 overexpression inhibited the apoptosis-inducing potential mediated by PI3K/Akt inhibition under ER stress, strongly suggest that PI3K/Akt protects HEK293 cells against ER stress-induced apoptosis through GRP78 regulation.

The expression of GRP78 is primarily regulated by ATF6, X-box binding protein 1 (XBP1), activating transcription factor 4 (ATF4) and p38 MAPK at the transcriptional level (Yoshida et al., 2001b; Luo and Lee, 2002; Li and Lee, 2006; Yamamoto et al., 2007). Our data showed that PI3K/Akt regulates GRP78 accumulation under ER stress at the protein level, but not at the transcriptional level. mTOR, an established target of Akt, regulates translation in response to nutrients and growth factors. Although ER stress-induced phosphorylation of mTOR was PI3K/Akt-dependent, it was not involved in GRP78 accumulation under ER stress. As GRP78 has been found to be a potential target of Akt phosphorylation in mesangial cells (Barati et al, 2006), it seems that PI3K/Akt can regulate the stability of GRP78 protein through phosphorylation. This speculation is supported by our demonstration that MG132 treatment blocked PI3K/Akt inhibition-mediated GRP78 protein reduction, but CHX had no effect on PI3K/Akt-mediated GRP78 regulation. Mechanistically, these results suggest that PI3K/Akt up-regulates GRP78 through increasing the stability of GRP78 protein under ER stress conditions. Akt established targets GSK3β and MDM2, which play pivotal roles in the regulation of protein stability, are not involved in the effect of Akt on GRP78 regulation. Further studies are needed to clarify the mechanisms by which Akt may contribute to increasing the stability of the GRP78 protein.

Mammalian cells express three different Akt isoforms, known as Akt1, Akt2, and Akt3, which share a high degree of amino acid identity and have a considerable functional overlap (Brazil et al, 2004). However, it is widely accepted that Akt1, Akt2 and Akt3 have physiologically diverse roles. It is notable that only Akt1, but not Akt2 or Akt3, is involved in GRP78 regulation in HEK293 cells under ER stress.

Although ER stress-mediated Akt gradual inactivation plays an important role in GADD153 induction (Hyoda et al., 2006), we found that PI3K/Akt had no appreciable effect on GADD153 accumulation in HEK293 cells under ER stress. Thus, our evidence indicates that GADD153 is not involved in the anti-apoptotic effects of PI3K/Akt in HEK293 cells under ER stress. Recently, it has been reported that ER stress can activate MEK/ERK rapidly, which in turn protects the cells against ER stress-induced apoptosis through promoting GRP78 induction (Jiang et al., 2007; Zhang et al., 2009). Here, we found that the phosphorylation levels of ERK1/2 rapidly decreased in dithiothreitol- and thapsigargin-treated HEK293 cells, and MEK/ERK inhibition had no effect on GRP78 induction. This suggests that MEK/ERK is not involved in ER stress-mediated GRP78 induction in HEK293 cells.

In this work, we reported that PI3K/Akt is required for ER stress-induced GRP78 accumulation in HEK293 cells, which consequently facilitates the cells to adapt to ER stress. However, PI3K/Akt inhibition in NIH 3T3 cells had no effect on ER stress-induced GRP78 expression, implying that the link between PI3K/Akt and GRP78 is cell-specific. As the activation of Akt decreased obviously within 6 h under ER stress, and GRP78 protein accumulated notably after 6 h exposure to ER stress in NIH 3T3 cells (Fig. 2E), it is reasonable that NIH 3T3 cells utilize other compensating pathways to stabilize GRP78. We also showed that only Akt1 is the predominant isoform which is involved in the regulation of GRP78 accumulation. We further found that PI3K/Akt promotes GRP78 accumulation at the protein level but not at the mRNA level. Further studies on the detailed mechanisms of PI3K/Akt-mediated GRP78 stability regulation will contribute to understanding the important role of the PI3K/Akt pathway under ER stress.

References

Anderson, K. E., Coadwell, J., Stephens, L. R., Hawkins, P. T. (1998) Translocation of PDK1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr. Biol.* **8**, 684-691.

Arai, K., Lee, S. R., Van Leyen, K., Kurose, H., Lo, E. H. (2004) Involvement of ERK MAP kinase in endoplasmic reticulum stress in SH-SY5Y human neuroblastoma cells. *J. Neurochem.* **89**, 232-239.

Barati, M. T., Rane, M. J., Klein, J. B., Mcleish, K. R. (2006) A proteomic screen identified stress-induced chaperone proteins as targets of Akt phosphorylation in mesangial cells. *J. Proteome Res*. **5**, 1636-1646.

Brazil, D. P., Yang, Z. Z, Hemmings, B. A. (2004) Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem. Sci.* **29**, 233-242.

Chung, J., Bachelder, R. E., Lipscomb, E. A., Shaw, L. M., Mercurio, A. M. (2002) Integrin (α6β4) regulation of eIF-4E activity and VEGF translation: a survival mechanism for carcinoma cells. *J. Cell Biol.* **158**, 165-174.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789.

Datta, K., Bellacosa, A., Chan, T. O., Tsichlis, P. N. (1996) Akt is a direct target of the phosphatidylinositol 3-kinase: Activation by growth factors, v-src and v-Ha-ras, in Sf9 and mammalian cells. *J. Biol. Chem.* **271**, 30835-30839.

Dong, D., Ni, M., Li, J., Xiong, S., Ye, W., Virrey, J. J., Mao, C., Ye, R., Wang, M., Pen, L., Dubeau, L., Groshen, S., Hofman, F. M., Lee, A. S. (2008) Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development. *Cancer Res.* **68**, 498-505.

Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., Tsichlis, P. N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**, 727-736.

Gething, M. J., Sambrook, J. (1992) Protein folding in the cell. *Nature* **355**, 33-45.

Hara, S., Nakashiro, K., Goda, H., Hamakawa, H. (2008) Role of Akt isoforms in HGF-induced invasive growth of human salivary gland cancer cells. *Biochem. Biophys. Res. Commun*. **370**, 123-128.

Harding, H. P., Zhang, Y., Ron, D. (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397**, 271-274.

Harding, H. P., Calfon, M., Urano, F., Novoa, I., Ron, D. (2002) Transcriptional and translational control in the mammalian unfolded protein response. *Annu. Rev. Cell Dev. Biol.* **18**, 575-599.

Hendershot, L. M. (2004) The ER function BiP is a master regulator of ER function. *Mt. Sinai J. Med.* **71**, 289-297.

Hosoi, T., Hyoda, K., Okuma, Y., Nomura, Y., Ozawa, K. (2007) Akt up- and down-regulation in response to endoplasmic reticulum stress. *Brain Res.* **1152**, 27-31.

Hu, P., Han, Z., Couvillon, A. D., Exton, J. H. (2004) Critical role of endogenous Akt/IAPs and MEK1/ERK pathways in counteracting endoplasmic reticulum stress-induced cell death. *J. Biol. Chem.* **279**, 49420-49429.

Hyoda, K., Hosoi, T., Horie, N., Okuma, Y., Ozawa, K., Nomura, Y. (2006) PI3K-Akt inactivation induced CHOP expression in endoplasmic reticulum-stressed cells. *Biochem. Biophys. Res. Commun.* **340**, 286-290.

Jiang, C. C., Chen, L. H., Gillespie, S., Wang, Y. F., Kiejda, K. A., Zhang, X. D., Hersey, P. (2007) Inhibition of MEK sensitizes human melanoma cells to endoplasmic reticulum stress-induced apoptosis. *Cancer Res*. **67**, 9750-9761.

Lee, A. S. (2005) The ER chaperone and signalling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods* **35**, 373-381.

Li, J., Lee, A. S. (2006) Stress induction of GRP78/BiP and its role in cancer. *Curr. Mol. Med.* **6**, 45-54.

Luo, S., Lee, A. S. (2002) Requirement of the p38 mitogen-activated protein kinase signalling pathway for the induction of the 78 kDa glucose-regulated protein/immunoglobulin heavy-chain binding protein by azetidine stress: activating transcription factor 6 as a target for stress-induced phosphorylation. *Biochem. J.* **366**, 787-795.

Marte, B. M., Downward, J. (1997) PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem. Sci.* **22**, 355-358.

Mayo, L. D., Donner, D. B. (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc. Natl. Acad. Sci. USA* **1998**, 11598-11603.

Mori, K. (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* **101**, 451-454.

Rutkowski, D. T., Kaufman, R. J. (2004) A trip to the ER: coping with stress. *Trends Cell Biol.* **14**, 20-28.

Schröder, M., Kaufman, R. J. (2005) ER stress and the unfolded protein response. *Mutat. Res.* **569**, 29-63.

Srinivasan, S., Ohsugi, M., Liu, Z., Fatrai, S., Bernal-Mizrachi, E., Permutt, M. A. (2005) Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3β in mouse insulinoma cells. *Diabetes* **54**, 968-975.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R., Reese, C. B., McCormick, F., Tempst, P., Coadwell, J., Hawkins, P. T. (1998) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* **279**, 710-714.

Wendel, H. G., De Stanchina, E., Fridman, J. S., Malina, A., Ray, S., Kogan, S., Cordon-Cardo, C., Pelletier, J., Lowe, S. W. (2004) Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* **428**, 332-337.

Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A., Mori, K. (2007) Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6α and XBP1. *Dev. Cell* **13**, 365-376.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., Mori, K. (2001a) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107**, 881-891.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., Mori, K. (2001b) Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6α and 6β that activates the mammalian unfolded protein response. *Mol. Cell Biol.* **21**, 1239-1248.

Zhang, L. J., Chen, S., Wu, P., Hu, C. S., Thorne, R. F., Luo, C. M., Hersey, P., Zhang, X. D. (2009) Inhibition of MEK blocks GRP78 up-regulation and enhances apoptosis induced by ER stress in gastric cancer cells. *Cancer Lett.* **274**, 40-46.