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PI3K-Akt inactivation induced CHOP expression in Endoplasmic reticulum-stressed cells

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Running title: Inactivation of Akt triggered CHOP induction

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Abstract

Stress signals that impair the function of the endoplasmic reticulum (ER) can lead to an accumulation of unfolded proteins in the ER causing cell death. Recent studies have indicated that ER stress contributes to several diseases such as neurodegenerative disorders or diabetes. In the present study, we found that Akt down-regulation is important for inducing CHOP expression, an ER stress-induced transcription factor. Treatment with tunicamycin or thapsigargin, ER stress inducers, caused dephosphorylation of Akt from 12 to 24 h and induced cell death. Interestingly, treatment with a PI3K inhibitor alone induced CHOP expression and caused cell death. However, a MEK1 inhibitor induced neither CHOP expression nor cell death. These results indicate that the inactivation of Akt by ER stress induces CHOP expression and causes cell death. Therefore, Akt plays an important role in ER stressed condition and may have important implications for understanding ER stress-related diseases.

Keywords: endoplasmic reticulum stress; PI3K-Akt pathway; LY294002; wortmannin; PD98059; CHOP

Abbreviations: CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; ERK, extracellular signal-regulated protein kinase; JNK, c-jun NH₂-terminal kinase; LDH, lactate dehydrogenase; PDK1/2, 3-phosphoinositide-dependent kinase 1/2; PI3K, phosphatidylinositide-3-OH kinase; PKB, protein kinase B; Tg, thapsigargin; Tm, tunicamycin; UPR, unfolded protein response

Introduction

Recent studies have shown that neurodegenerative disorders such as Alzheimer's and Parkinson's disease are involved in a disruption of endoplasmic reticulum (ER) function [1, 2]. In response to ER stress, unfolded proteins accumulate and aggregate in the ER, resulting in activation of the unfolded protein response (UPR) [3]. Although moderate stress acting on the ER will trigger a variety of rescuer responses, excessive or long-term stress acting on the ER will induce cell death. Recently, several mechanisms of ER stress-mediated signaling have been proposed, e.g. 1) ER stress-induced induction of the apoptotic transcription factor CHOP [4, 5, 6], 2) activation of caspase-12 [7, 8] and 3) activation of c-jun NH₂-terminal kinase (JNK)-mediated pathways [9]. On the other hand, ER stress activates NF- κ B- or GRP78-mediated pathways, which protects cell death [10, 11].

Insulin and several growth factors promote cell survival by activating phosphatidylinositol-3-OH kinase (PI3K) [12, 13]. One of the downstream targets of PI3K is a serine/threonine kinase, Akt/protein kinase B (PKB). 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 [14, 15, 16].

There are many signaling pathways from each organelle that cause cell death. Mitochondria-mediated apoptotic proteins, such as Bad and caspase-9, are well known [17]. It has been reported that Akt phosphorylates and inactivates these proteins [18, 19, 20]. Recently, insulin-like growth factor-1 (IGF-1) has been shown to reduce thapsigargin (an ER stress inducer)-induced apoptosis in insulinoma cells [21]. However, it is unclear whether Akt participates in ER stress-mediated signaling. Since

Akt has been shown to promote cell survival against several death signals, we investigated whether its activation is regulated by ER stress. In the present study, we observed that Akt was gradually inactivated in response to long-term exposure to ER stress. Importantly, inactivation of Akt alone induced CHOP expression and caused cell death. The results therefore suggest that Akt inactivation is involved in ER stress-induced cell death.

Materials and methods

Materials and reagents. Tunicamycin (Tm) and thapsigargin (Tg) were obtained from Wako Pure Chemical Ltd. (Japan). LY294002 and wortmannin were purchased from SIGMA (St. Louis, MO). PD98059 was provided by RBI (MA).

Cell culture. Mouse fibroblast L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and antibiotics (100 units/ml penicillin G and 100 µg/ml streptomycin; GIBCO BRL) at 37°C in humidified 5% CO₂, 95% air. L929 cells were transferred to a serum-free medium before the lactate dehydrogenase (LDH) assay and Western blotting.

LDH assay. The viability of L929 cells after Tm or Tg treatment was estimated by measuring LDH leakage using a cytotoxicity detection kit (Roche Molecular Biochemical) according to the manufacturer's protocol. LDH activity was measured as optical density at 492 nm.

RT-PCR. Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO). RT-PCR was performed as described previously [22]. Precisely, cDNA was synthesized from total RNA by reverse transcription using 100 U of Superscript Reverse Transcriptase (GIBCO BRL) and Oligo (dt)₁₂₋₁₈ primer (GIBCO BRL) in a 20 µl reaction mixture containing Superscript buffer (GIBCO BRL), 1 mM dNTP mix, 10 mM DTT, and 40 U of RNase inhibitor. Total RNA and Oligo (dt)₁₂₋₁₈ primer were

incubated at 70°C for 10 min prior to the reverse transcription. After incubation for 1 h at 42°C, the reaction was terminated by a denaturing enzyme for 15 min at 70°C. For PCR amplification, 1.2 µl of cDNA was added to 12 µl of a reaction mix containing 0.2 µM of each primer, 0.2 µM of dNTP mix, 0.6 U of Taq polymerase, and reaction buffer. PCR was performed in a DNA Thermal Cycler (GeneAmp® PCR System 9700). The following primers were used: CHOP upstream, 5'-ccc tgc ctt tca cct tgg-3'; CHOP downstream, 5'-ccg ctc gtt ctc ctg ctc-3'; GAPDH upstream, 5'-aaa ccc atc acc atc ttc cag -3'; and GAPDH downstream, 5'-agg ggc cat cca cag tct tct-3'. The PCR products (10 µL) were resolved by electrophoresis in an 8% polyacrylamide gel in TBE buffer. The gels were stained with ethidium bromide and then photographed under ultraviolet light. cDNA for GAPDH and CHOP were amplified for 18 (94°C 1 min, 57°C 1 min, 72°C 1 min) and 20 (94°C 1 min, 60°C 1 min, 72°C 1 min) cycles, respectively, and these PCR reactions were run separately. These cycle numbers were chosen based on a preliminary study determining the linear range of amplification for each respective molecule. To compare the expression of mRNAs in the different experimental groups, the amount of mRNA in each structure studied was estimated as the ratio of CHOP/GAPDH.

Western blotting. Western blotting was performed as described previously [23]. Cells were washed with ice-cold PBS and lysed in a buffer containing 10 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM Na₃VO₄, 10 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and 1% NP-40 for 20 min. The lysates were centrifuged at 15,000 rpm for 20 min at 4°C, and the supernatants were collected. The samples were boiled with laemmli buffer for 3 min, fractionated by

SDS-PAGE, and transferred at 4°C to nitrocellulose membranes. The membranes were incubated with anti-phospho-Akt (Thr308 and Ser473: Cell Signaling; diluted 1:1,000), anti-Akt (Cell Signaling; 1:1,000), anti-phospho-ERK (Thr202/Tyr204: Cell Signaling; 1:1,000) and anti-CHOP (Santa Cruz; 1:500) antibodies and then with anti-horseradish peroxidase-linked antibody. Peroxidase was detected by chemiluminescence using an ECL system (Amersham).

Results and discussion

ER stress down-regulated Akt activation.

We investigated whether ER stress induces cell death in L929 cells using the LDH assay. ER stress-inducing reagents, Tm (N-glycosylation inhibitor; 3 $\mu\text{g}/\text{mL}$) and Tg (sarco/ER Ca^{2+} ATPase inhibitor; 3 μM), slightly induced cell death at 12 h, and a marked increase was observed at 24, 36 and 48 h (Fig. 1A). CHOP has been shown to be involved in ER stress-induced cell death [4, 5, 6]. Thus, we analyzed CHOP induction. As assessed by Western blotting, CHOP was induced at 12-24 h after Tm (3 $\mu\text{g}/\text{mL}$) or Tg (3 μM) treatment (Fig. 1B). These results suggest that ER stress induces expression of CHOP and causes cell death in L929 cells.

To explore the possible involvement of Akt in ER stress, we next examined Akt phosphorylation in stressed cells by Western blotting. We found that basal level of Akt phosphorylation was gradually dephosphorylated by long-term exposure to ER stress in L929 cells (Figs. 2A, 2B and 2C). We observed a slight decrease in phosphorylation at 12 h, and Akt was completely dephosphorylated at 24 h in response to Tm (3 $\mu\text{g}/\text{mL}$) or Tg (3 μM). On the other hand, the expression level of total Akt protein was not changed in response to ER stress (Figs. 2A and 2D), indicating that the down-regulation of Akt phosphorylation was not due to the down-regulation of normal Akt protein level. Akt down-regulation precedes cell death (compare Fig. 1A and Fig. 2A) and the results raised the possibility that Akt is a physiologically important molecule in the regulation of ER stress-induced cell death.

Inhibition of PI3K-Akt induced CHOP expression.

It has been demonstrated that CHOP and GRP78 are induced in response to ER stress [4, 5, 6, 11]. Since we observed that ER stress down-regulated Akt activation, we next investigated whether down-regulation of the PI3K-Akt pathway affects these molecules. L929 cells were treated with PI3K inhibitors (LY294002 and wortmannin) and Western blotting was performed. Treatment with LY294002 (Figs. 3 and 4) or wortmannin (data not shown) inhibited basal Akt phosphorylation. Surprisingly, under these conditions, treatment with a PI3K inhibitor alone induced CHOP expression at both mRNA and protein levels. Expression of CHOP mRNA was induced 2 h after LY294002 or wortmannin treatment (Fig. 5). Time course experiments indicated that CHOP protein was induced from 6 to 24 h after LY294002 application (Fig. 3). Dose-dependent induction of CHOP was observed after LY294002 (20-50 μ M) treatments (Figs. 3, 4 and 5). On the other hand, we did not observe GRP78 induction (data not shown), suggesting that PI3K-Akt inhibition specifically induces CHOP expression. As shown in the Figures 1 and 2, ER stress-induced CHOP expression appears to precede Akt down-regulation. However, it has been demonstrated that ER stress responses consist of multiple phases [24]. Therefore, it is speculated that inactivation of PI3K-Akt pathway is involved in late phase of ER stress responses which induces CHOP expression.

It has been reported that PI3K activity is essential for activation of the Raf/MEK1/ERK cascade [25, 26]. LY294002, a specific PI3K inhibitor, suppressed Akt phosphorylation and at the same time, we observed suppression of basal ERK phosphorylation (Fig. 4). Thus, it is possible that inhibition of ERK is involved in the CHOP induction. However, PD98059 (20 μ M), a MEK1 inhibitor, did not induce CHOP expression (Figs. 4 and 5). These results indicate that inactivation of the

MEK1-ERK pathway is not involved in CHOP induction. It is suggested that CHOP is induced specifically through inhibition of the PI3K-Akt pathway.

Inhibition of PI3K-Akt caused cell death.

Since we observed that inactivation of PI3K-Akt induced CHOP expression, we next examined whether inactivation of PI3K-Akt induces cell death. L929 cells were treated with LY294002 (20-50 μ M) and PD98059 (20 μ M) for 24-48 h, and LDH assay was performed. Interestingly, as shown in Figure 6, we observed a dose- and time-dependent increase in cell death after LY294002 treatment (Fig. 6). However, PD98059 did not induce cell death at neither time point (24-48 h) determined. Thus, it is suggested that inactivation of PI3K-Akt specifically induces cell death. As PD98059 did not induce CHOP expression (Figs. 4 and 5), PI3K-Akt inactivation-induced cell death may be mediated through CHOP induction.

Taken together, ER stress-induced Akt inactivation may cause cell death by inducing CHOP expression and that inhibition of the PI3K-Akt pathway alone may be sufficient for inducing CHOP. The present findings may have important implications for understanding ER stress-mediated disorders such as neurodegenerative diseases or diabetes.

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Figure legends

Fig. 1. ER stress induced CHOP expression and caused cell death in mouse fibroblast L929 cells. L929 cells were treated with Tm (3 $\mu\text{g}/\text{mL}$) or Tg (3 μM) for the periods indicated. A, LDH assay was performed at the time point indicated. The amount of LDH released into the medium was expressed as a percentage of the control value. Values are presented as means \pm S.E. for three separate experiments. B, CHOP was detected by Western blotting using a specific antibody.

Fig. 2. ER stress down-regulated Akt activation. L929 cells were treated with Tm (3 $\mu\text{g}/\text{mL}$) or Tg (3 μM) for the indicated times. A, Phospho-Akt (Thr308 and Ser473) and total Akt levels were detected by Western blotting. B, C and D, Densitometric analysis using image analyzing software. The expression levels are expressed as fold intensity compared with unstressed cells. Values are presented as means \pm S.E. for three separate experiments.

Fig. 3. LY294002 induced CHOP expression. A, L929 cells were treated with LY294002 (20-50 μM) for the indicated times. CHOP, phospho-Akt (Ser473) and total Akt levels were analyzed by Western blotting. B, Densitometric analysis using image analyzing software. The expression levels are expressed as fold intensity compared with the control treated group. Values are presented as means \pm S.E. for three separate experiments.

Fig. 4. ERK was not required for CHOP induction. A, L929 cells were treated with

LY294002 (LY, 20-50 μ M) or PD98059 (PD, 20 μ M) for 24 h. CHOP, phospho-Akt (Ser473), total Akt and phospho-ERK (Thr202/Tyr204) levels were analyzed by Western blotting. B, Densitometric analysis using image analyzing software. The expression levels are expressed as fold intensity compared with the control treated group. Values are presented as means \pm S.E. for three separate experiments.

Fig. 5. Inactivation of PI3K-Akt induced CHOP mRNA expression. A, L929 cells were treated with LY294002 (LY, 20-50 μ M), wortmannin (wt, 500 nM) or PD98059 (PD, 20 μ M) for 2 h, and RT-PCR was performed. B, The amounts of CHOP mRNA are expressed as the ratio of densitometric measurements of the samples to the corresponding GAPDH internal control. Values are presented as means \pm S.E. for three separate experiments.

Fig. 6. Inactivation of PI3K-Akt caused cell death. L929 cells were treated with LY294002 (LY, 20-50 μ M) or PD98059 (PD, 20 μ M) for the periods indicated, and LDH assay was performed. The amount of LDH released into the medium was expressed as a percentage of the control value. Values are presented as means \pm S.E. for three separate experiments.