PKC ι counteracts oxidative stress by regulating Hsc70 in an esophageal cancer cell line

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Abstract Using a glutathione *S*-transferase pull-down liquid chromatography–coupled tandem mass spectrometry approach and immunoprecipitation/immunoblot analysis, we found that heat shock cognate protein 70 (Hsc70) was involved in the complex formed by atypical protein kinase C ι (PKC ι) and LC3 in the esophageal cancer cell line KYSE30. Further study indicated that Hsc70 was targeted by autophagic degradation, and knockdown of PKC ι down-regulated Hsc70 by promoting autophagy. PKC ι knock-down sensitized cells to oxidative stress-induced apoptosis, whereas forced PKC ι expression counteracted the oxidative stress-induced apoptosis via Hsc70.

Keywords PKC ι · Hsc70 · Oxidative stress · Autophagic degradation

Abbreviation

LC	Liquid chromatography
MS	Mass spectrometry
PKC ι	Atypical protein kinase C ι
Hsc70	Heat shock cognate protein 70

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Introduction

Atypical protein kinase C ι (PKC ι) has been identified as an oncoprotein in lung cancer (Regala et al. 2005a, b), ovarian cancer (Eder et al. 2005), gastric cancer (Takagawa et al. 2010), colon cancer (Murray et al. 2004), and esophageal squamous cell carcinoma (ESCC) (Yang et al. 2008). PKC ι regulates multiple malignant phenotypes and several oncogenic signaling pathways in cancer cells. In chronic myelogenous leukemia, PKC ι regulates Bcr-Abl-induced drug resistance (Jamieson et al. 1999). In colon cancer, this kinase is required for Ras-mediated transformation (Murray et al. 2004), whereas in lung cancer, it phosphorylates μ -calpain and m-calpain and is involved in nicotine-induced carcinogenesis (Xu and Deng 2006). Moreover, our laboratory previously investigated the roles of PKC ι in esophageal cancer cells and reported that PKC ι promotes anoikis resistance and metastasis by activating the SKP2-Akt axis (Liu et al. 2011).

Heat shock cognate protein 70 (Hsc70; also known as heat shock cognate 71-kDa protein, HspA8 and Hsp73) is a constitutively expressed member of the Hsp70 family (Dworniczak and Mirault 1987; Daugaard et al. 2007). Hsc70 participates in several cellular processes, including viral replication (Watanabe et al. 2006; Hayashi et al. 2009), viral infection (Parent et al. 2009), and autoimmune inflammation (Alam et al. 2009). Hsc70 functions as a chaperone by trafficking proteins to different cellular compartments (Pilon and Schekman 1999) and is important in endocytosis (de Waegh and Brady 1989). Hsc70 is also involved in Akt (Shiota et al. 2010; Chen et al. 2011) and NF- κ B signaling (Lim et al. 2008) and has been shown to regulate the Dbl oncoprotein (Kauppinen et al. 2005) and Bim mRNA (Matsui et al. 2007). Importantly, Hsc70 also regulates cell survival (Powers et al. 2008) and confers protection from

several forms of cellular stresses, such as viral infection (Yan et al. 2010), metabolic stress (Williams et al. 1993), and oxidative stress (Chong et al. 1998; Dastoor and Dreyer 2000; Su et al. 1999). Thus far, the mechanism of regulation of Hsc70 remains unclear, but it appears that its expression is regulated by KLF4 (Liu et al. 2008). In the present study, we found that PKC ϵ served as a regulator of Hsc70 in an esophageal cancer cell line and that PKC ϵ regulates oxidative sensitivity via Hsc70.

Materials and methods

Cell culture and transfection

The human ESCC cell line KYSE30 was generously provided by Dr. Y Shimada (Kyoto University, Kyoto, Japan) (Shimada et al. 1992). The cells were cultured in RPMI 1640 (Invitrogen, San Diego, CA, USA) supplemented with 10 % fetal bovine serum. Cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Reagents and antibodies

Bafilomycin (BFA1), 3-methyladenine (3MA), and rapamycin were purchased from Sigma-Aldrich, St. Louis, MO, USA. H₂O₂ was purchased from Applygen Technologies. The primary antibodies used in this work included anti-PKC ϵ (1:1,000; BD PharMingen, San Diego, CA, USA), anti-Hsc70 (1:1,000; Proteintech Group, Inc., Chicago, IL, USA), anti-LC3 (1:1,000; MBL International, Woburn, MA, Japan), anti- β -actin (1:5,000; Sigma), and anti-p62 (1:1,000; MBL International, Woburn, MA, Japan).

Plasmid construction and small interfering RNA synthesis

The PKC ϵ expression vector was constructed, and the PKC ϵ siRNAs were designed as previously described (Liu et al. 2011). The duplex siRNAs were chemically synthesized by the GenePharma Company (Shanghai, China) with the following sequences: PKC ϵ siRNA, 5'-AAGCCTGGATACAATTAACCA-3'; ATG5 siRNA, 5'-GACGTTGGTAACTGACAAA-3'; Lamp2a siRNA, 5'-GCTCTACTTAGACTCAATA-3'; P62 siRNA-1, CAGATGGAGTCCGATAACT; Hsc70 siRNA, 5'-GCTGGTCTCAATGTACTTA-3'; and control siRNA, 5'-TTCTCCGAACGTGTCACGT-3' (negative control).

GST pull-down

The recombinant glutathione *S*-transferase (GST)-tagged PKC ϵ (aa 523–594)/LC3 (full-length) fusion protein

expressed in *Escherichia coli* was affinity-purified and subsequently immobilized on glutathione affinity resin (Pierce). GST-tagged PKC ϵ /LC3 resin or the control (GST resin) was incubated for 3 h at 4 °C with 5 mg of the total protein. After washing the resin, the bound proteins were eluted, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and visualized by zinc staining using a Pierce Zinc Reversible Stain kit (Thermo Scientific). The bands present only in the GST-LC3B resin eluates were excised, digested with trypsin, and then subjected to liquid chromatography–coupled tandem mass spectrometry (LC/MS/MS) and peptide identification.

LC/MS/MS and peptide identification

An Agilent 1100 HPLC system was connected to an LTQ Orbitrap Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The instrument was equipped with a 20-mm $\times$ 100- μ m i.d. Aqua C18 trap column (Phenomenex, Torrance, CA, USA) and a 200-mm $\times$ 50- μ m i.d. Reprosil C18 RP analytical column (Dr. Maisch, Ammerbuch-Entringen, Germany). The tryptic peptides were separated by using a 90-min 200-nL/min linear gradient from 0 to 60 % solvent B (0.1 M acetic acid in 80 % acetonitrile (v/v)), in which solvent A was 0.1 M acetic acid. The MS was operated in positive ion mode, and parent ions were isolated for fragmentation in data-dependent mode.

The search parameters were set as follows: taxonomy; database, NCBI/IPI; enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); max missed cleavages, one/two; mass tolerances for MS and MS/MS were 10 ppm and 0.5 Da, respectively.

Immunoprecipitation

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with lysis buffer using a Roche Immunoprecipitation kit. The total protein concentration was estimated using a Protein Assay kit (Bio-Rad, Richmond, CA, USA). Total protein (5 mg) was incubated with 50 μ L of a protein G-agarose suspension (Roche Applied Science, Indianapolis, IN, USA) for 3 h at 4 °C on a rocking platform to reduce non-specific binding. After removing the beads, the supernatant was supplemented with 5 μ g of anti-LC3 antibody (MBL International), 5 μ g of anti-p62 antibody (MBL International), or 5 μ g of anti-PKC ϵ antibody (BD PharMingen), and the cells were then incubated for an additional 3 h at 4 °C. A total of 50 μ L of protein G-agarose was then added to each immunoprecipitation mixture, and the incubation was continued overnight at 4 °C on a rocking platform. The immunoprecipitates were collected by centrifugation and washed

three times with the wash buffer. After loading buffer was added, the agarose was boiled and subjected to western blot analysis.

Western blot analysis

Cells were washed with ice-cold PBS and lysed in RIPA buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS] containing phenylmethylsulfonyl fluoride (1 mmol/L) and protease inhibitors (2 g/mL; Protease Inhibitor Cocktail Set III, Calbiochem) on ice for 30 min. The lysates were clarified by centrifugation at 13,000 $\times$ g for 30 min at 4 °C. The total protein concentration was estimated using a Protein Assay kit (Bio-Rad). Protein samples (50–150 μ g) were separated using SDS–PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Temecula, CA, USA), and then blocked and probed with primary antibodies. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using the super-ECL detection reagent (Applygen Technologies, Inc., Beijing, China).

Flow cytometry assay

Cells were harvested to assay apoptosis using an Annexin V-FITC apoptosis detection kit (Sigma) at 72 h after transfection. The cells were incubated with Annexin V for 30 min and then with PI for 5 min, and the cells were subsequently analyzed using flow cytometry. The apoptotic cells were a combination of early apoptotic cells (Annexin V-positive and PI-negative) and late-apoptotic cells (Annexin V-positive and PI-positive).

Caspase-3 activity assay

Cells were harvested to assay caspase-3 activity using a Caspase-Glo[®] 3/7 Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical analysis

The differences in the results between groups were compared using the Student's *t* test. Data are expressed as the mean $\pm$ SEM.

Results

Hsc70 interacts with the PKC ι –LC3 complex

By screening proteins that interacted with PKC ι and LC3, we consistently found that Hsc70 interacted with both PKC ι

and LC3 in KYSE30 esophageal cancer cells. We constructed GST-PKC ι (aa 1–253), GST-PKC ι (aa 523–594), and GST-LC3 (full-length) fusion proteins as bait to purify binding proteins from the lysates of KYSE30 cells. The proteins that were pulled down were resolved by SDS–PAGE (Fig. 1a, b), and the bands that were present only in the GST-PKC ι /LC3 resin eluates were excised, digested with trypsin, and subjected to LC/MS/MS analysis. The results showed that Hsc70 was pulled down by both the GST-PKC ι (aa 523–594) and GST-LC3 fusion proteins (Supplementary Table 1). The presence of Hsc70 in the GST-PKC ι (aa 523–594) and GST-LC3 resin eluates was further confirmed by western blot (Fig. 1a, b), and the interaction between Hsc70 and PKC ι and LC3 was also confirmed by immunoprecipitation using anti-PKC ι and anti-LC3 antibodies. These results demonstrated that Hsc70 could be co-immunoprecipitated with both PKC ι and LC3 (Fig. 2a, b). PKC ι is known to interact with LC3 (Behrends et al. 2010), and our experiments supported this finding (Fig. 2a, b). Therefore, Hsc70, PKC ι , and LC3 were present in the same complex in KYSE30 cells. p62 has also been shown to be a bona fide partner of PKC ι and LC3 (Sanchez et al. 1998; Sanz et al. 1999; Behrends et al. 2010; Johansen and Lamark 2011), and using an immunoprecipitation assay, we found that Hsc70 also co-immunoprecipitated with p62 (Fig. 2c). The interaction between Hsc70 and LC3 was promoted by p62, as the knockdown of p62 reduced this interaction (Fig. 2d).

PKC ι regulates Hsc70 in an autophagy-dependent manner

The observation that Hsc70 interacted with LC3 and this was mediated by p62 suggested that Hsc70 was a potential target of autophagic degradation, as proteins degraded through this pathway need to be recognized by the LC3–p62 complex (Johansen and Lamark 2011; Lamark et al. 2009). To test this possibility, we used rapamycin to induce autophagy and found that Hsc70 was down-regulated by rapamycin but that this effect could be blocked by the autophagy inhibitor 3MA (Fig. 3a). Together, these results suggest that Hsc70 is regulated by autophagy. PKC ι serves as a negative regulator of autophagy, as it has been shown to interact with and phosphorylate LC3 and also inhibit LC3 processing and autophagosome formation (Behrends et al. 2010; Jiang et al. 2010). Thus, we hypothesized that PKC ι regulates Hsc70 by inhibiting autophagy. To test this hypothesis, we inhibited PKC ι expression using siRNA and found that knockdown of PKC ι expression enhanced cellular autophagy and down-regulated Hsc70 (Fig. 3b, c, columns 1 and 2). Moreover, the disruption

Fig. 1 Identification of Hsc70 as a PKC ϵ - and LC3-binding protein. GST-PKC ϵ (aa 523–594) (**a**) and GST-LC3 fusion proteins (**b**) were immobilized on glutathione-Sepharose beads and incubated with KYSE30 cell lysates. Following GST pull-down, the purified proteins were resolved by SDS-PAGE and visualized by zinc staining. Western blot assay confirmed the presence of Hsc70 in GST-PKC ϵ and GST-LC3 resin eluates

of autophagy by ATG5 knockdown prevented the down-regulation of Hsc70 induced by PKC ϵ inhibition (Fig. 3b, columns 3 and 4). In addition, the down-regulation of Hsc70 caused by PKC ϵ knockdown was also blocked following BFA1 treatment (Fig. 3c, columns 3 and 4), which interferes with the fusion of the autophagosome to the lysosome and inhibits the degradation of autophagic substrates through the autophagy–lysosome pathway (Pan et al. 2008). Upon BFA1 treatment, knockdown of PKC ϵ

promoted the interaction between Hsc70 and LC3 and P62 (Fig. 3d, e). These results suggest that depletion of PKC ϵ promoted the autophagic degradation of Hsc70. Although Hsc70 is a receptor protein in the chaperone-mediated autophagy (CMA) pathway, our results demonstrate that the regulation of Hsc70 by PKC ϵ was CMA-independent, as impairment of CMA following the knockdown of Lamp2a (Massey et al. 2006; Liu et al. 2009) did not block the regulation of Hsc70 by PKC ϵ (Fig. 3f).

Fig. 2 Co-immunoprecipitation of Hsc70 with PKC ϵ , LC3, and p62. Immunoprecipitation assays using anti-PKC ϵ (**a**), anti-LC3 (**b**), or anti-p62 (**c**) antibodies demonstrated that Hsc70 could be co-immunoprecipitated with PKC ϵ , LC3, and p62. **d** KYSE30 cells were transfected with control siRNA or p62 siRNA for 48 h. Then, the cells were collected for immunoprecipitation using the anti-LC3 antibody and immunoblotted with the indicated antibodies

Fig. 3 PKC ι positively regulates Hsc70 in an autophagy-dependent manner. **a** Western blot analysis confirmed the down-regulation of Hsc70 in cells that were treated with the autophagy inducer rapamycin (0.2 μ mol/L) for 24 h; this effect was reversed following 3MA treatment (10 mmol/L). **b** KYSE30 cells were transfected with control siRNA or PKC ι siRNA. Prior to analysis, the cells were cultured in normal media for 48 h, and then the cells were either left untreated (control) or treated with BFA1 (0.1 μ mol/L) for 24 h. Next, the cells were harvested and subjected to western blot analysis using the indicated antibodies. The results show that knockdown of PKC ι down-regulated Hsc70 protein but that BFA1 treatment inhibited the down-regulation of Hsc70 following PKC ι siRNA transfection. **c** KYSE30 cells were transfected with the following: control siRNA (80 nmol/L), control siRNA (40 nmol/L) and PKC ι siRNA (40 nmol/L), ATG5 siRNA (40 nmol/L) and control siRNA (40 nmol/L), or ATG5 siRNA (40 nmol/L) and PKC ι siRNA (40 nmol/L). Then, 72 h after transfection, the cells were harvested and subjected to western blot analysis

using the indicated antibodies. The results indicated that the down-regulation of Hsc70 was inhibited as a result of the disruption of autophagy through ATG5 siRNA transfection. **d**, **e** KYSE30 cells were transfected with control siRNA or PKC ι siRNA. Prior to analysis, the cells were cultured in normal media for 48 h and were then treated with BFA1 (0.1 μ mol/L) for 24 h. The cells were then collected for immunoprecipitation using the anti-LC3 or anti-p62 antibody and immunoblotted with the indicated antibodies. The results indicated that knockdown of PKC ι promoted the interaction between Hsc70 and the LC3–p62 complex. **f** Western blot analysis of Hsc70, PKC ι , and Lamp2a in KYSE30 cells 72 h after they were transfected with the following: control siRNA (80 nmol/L), control siRNA (40 nmol/L) and PKC ι siRNA (40 nmol/L), Lamp2a siRNA (40 nmol/L) and control siRNA (40 nmol/L), or Lamp2a siRNA (40 nmol/L) and PKC ι siRNA (40 nmol/L). These results indicated that depletion of PKC ι -induced Hsc70 down-regulation could not be rescued by Lamp2a knockdown

PKC ι protects the cell against oxidative stress-induced apoptosis through Hsc70

Hsc70 has been shown to protect cells from oxidative stress (Su et al. 1999; Chong et al. 1998), and we demonstrated

that PKC ι served as a positive regulator of Hsc70. Therefore, we hypothesized that PKC ι also regulated this oxidative protection mediated by Hsc70. We found that knockdown of PKC ι sensitized cells to H₂O₂-induced apoptosis, as measured by Annexin-V staining (Fig. 4a).

Moreover, over-expression of PKC ϵ up-regulated Hsc70 and significantly reduced H₂O₂-induced apoptosis, whereas knockdown of Hsc70 eliminated the protective role of PKC ϵ (Fig. 4b). These results demonstrate that PKC ϵ protects cells from oxidative stress-induced apoptosis by positively regulating Hsc70.

Discussion

Oxidative stress plays dual roles in cancer cell survival during tumorigenesis, as sublethal oxidative stress can activate oncogenic signals but severe oxidative stress can cause cell death or senescence (Liou and Storz 2010; Brown and Bicknell 2001). To survive and expand under exogenous or intrinsic oxidative stress, cancer cells co-opt several cellular regulatory pathways controlling oxidative adaptation to maintain cell survival. Reactive oxygen species (ROS) generation can be stimulated by the alteration of metabolic pathways, inadequate nutrient supply, or the activation of certain oncogenic proteins, including oncogenic Ras (Irani et al. 1997) and Akt (Nogueira et al. 2008). On the other hand, cancer-related proteins have also been shown to protect cells from ROS. For example, FoxM1 down-regulates ROS and maintains cell survival and growth under oxidative stress by stimulating ROS scavengers and Bim1 expression

(Park et al. 2009; Li et al. 2008). In addition, PKR-like endoplasmic reticulum kinase has a role in limiting oxidative damage (Bobrovnikova-Marjon et al. 2010). PKC ϵ is an oncoprotein in esophageal squamous cell carcinoma (Yang et al. 2008), and one of its roles is to promote cancer cell metastasis (Liu et al. 2011). The present work demonstrated an additional role for this oncoprotein in counteracting oxidative stress-induced apoptosis in esophageal cancer cells. These findings suggest that high PKC ϵ expression in esophageal cancer cells elevates the threshold of ROS tolerance and facilitates cell survival under oxidative stress.

We also observed that PKC ϵ regulated oxidative sensitivity by positively regulating Hsc70, a pro-survival protein that confers oxidative protection (Chong et al. 1998; Su et al. 1999). We found that Hsc70 is involved in a complex formed by PKC ϵ -p62/LC3 that functions in autophagic protein degradation (Behrends et al. 2010). As a protein degradation pathway, autophagic degradation participates in the regulation of cell signaling by selectively targeting signal proteins as well as contributing to the bulk clearance of cytoplasmic compartments (Yu et al. 2006; Komatsu et al. 2010; Gao et al. 2010; Yu et al. 2008). The observed interaction between Hsc70 and the receptor–adaptor system (p62/LC3) makes Hsc70 a potential substrate for this degradation machinery. PKC ϵ appears to regulate Hsc70 through complexation with p62/LC3-Hsc70 and inhibits

Fig. 4 PKC ϵ protects cells from oxidative stress-induced apoptosis by regulating Hsc70. **a** KYSE30 cells were transfected with control siRNA (80 nmol/L) or PKC ϵ siRNA (80 nmol/L), and at 36 h after transfection, the cells were treated without or with H₂O₂ (60 μ mol/L) for an additional 12 h. The cells were then harvested for Annexin-V/PI staining and flow cytometry analysis. **b** KYSE30 cells were transfected

with control siRNA (80 nmol/L) and empty vector (800 ng/mL), control siRNA (80 nmol/L) and PKC ϵ expression vector (800 ng/mL), or Hsc70 siRNA (80 nmol/L) and PKC ϵ expression vector (800 ng/mL). Thirty-six hours after transfection, the cells were treated with H₂O₂ (60 μ mol/L) for an additional 12 h. Then, the cells were harvested for caspase-3 activity analysis

LC3 processing and autophagosome formation (Jiang et al. 2010), thereby preventing the autophagic degradation of Hsc70. Conversely, when the expression of PKC ι is inhibited, cellular autophagy becomes activated, and Hsc70 is recruited by p62/LC3 and targeted for autophagic degradation. These findings not only reveal a potential mechanism that may be employed by cancer cells to counteract oxidative stress but also provide clues for therapeutic strategies based on targeting ROS production. In other words, the down-regulation of PKC ι may sensitize cancer cells to treatments aimed at generating oxidative stress.

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Conflict of interest The authors declare no conflict of interest.

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