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Cytoprotective roles of ERK and Akt in endoplasmic reticulum stress triggered by subtilase cytotoxin

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ABSTRACT

Subtilase cytotoxin (SubAB) is the prototype of a distinct AB_5 toxin family produced by Shiga toxigenic *Escherichia coli*. Recent reports disclosed pro-apoptotic pathways triggered by SubAB, whereas its anti-apoptotic signals have not been elucidated. In the present study, we investigated pro-survival signaling elicited by SubAB, especially focusing on extracellular signal-regulated kinase (ERK) and Akt. We found that SubAB activated ERK and Akt, and inhibition of individual kinases enhanced SubAB-triggered apoptosis. SubAB induced endoplasmic reticulum (ER) stress, and other ER stress inducers mimicked the stimulatory effects of SubAB on ERK and Akt. Attenuation of ER stress reduced SubAB-induced phosphorylation of these kinases, suggesting involvement of the unfolded protein response (UPR). SubAB induced activation of protein kinase-like ER kinase (PERK) and phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), and phosphorylation of eIF2α by salubrinal caused activation of ERK and Akt, leading to cell survival. Dominant-negative inhibition of PERK enhanced SubAB-induced apoptosis and reduced phosphorylation of ERK and Akt. Furthermore, the anti-apoptotic effect of eIF2α was significantly reversed by inhibition of ERK and Akt. These results suggest cytoprotective roles of ERK and Akt in SubAB-triggered, ER stress-mediated apoptosis.

1. Introduction

Shiga toxigenic *Escherichia coli* (STEC) is a serious pathogen in humans, because its infection results in life-threatening diseases such as hemolytic uremic syndrome (HUS) [1]. Shiga toxin, the pathotype-defining AB_5 toxin produced by STEC, comprises an A subunit with enzymatic activity and a B subunit pentamer responsible for interaction with glycolipid receptors [1,2]. Subtilase cytotoxin (SubAB), the prototype of a new family of AB_5 toxin, was discovered in a highly virulent STEC strain responsible for an outbreak of HUS and is produced by a number of other STEC strains [3,4]. Its B pentamer recognizes a distinct glycan receptor (N-glycolylneuraminic acid) displayed on cell surface glycoproteins, while it's A subunit is a protease that selectively cleaves the endoplasmic reticulum (ER) chaperone 78 kDa glucose-regulated protein (GRP78, also called BiP) [5,6]. *In vitro*, SubAB causes apoptosis of Vero cells and HeLa cells [7,8]. *In vivo*, injection of SubAB causes HUS-like pathologies in mice, which is associated with induction of apoptosis in the liver, kidney and spleen [9]. Currently, molecular mechanisms involved in these apoptotic processes are not fully addressed, but damage of the mitochondrial membrane and activation of pro-apoptotic Bax and Bak possibly play crucial roles [7,8,10]. In contrast, anti-apoptotic signaling elicited by SubAB has not been reported.

The mitogen-activated protein (MAP) kinase pathway is one of the most important and intensively investigated signaling pathway. It plays a central role in governing proliferation, differentiation and survival of many cell types, and its dysregulation is implicated in pathogenesis of various human diseases [11]. The mammalian MAP kinase family consists of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 MAP kinase. Upon activation, MAP kinases translocate from the cytoplasm to the nucleus, where they regulate gene transcription through affecting chromatin structure and modifying the activity of transcription factors. In the pathogenesis of Shiga toxin-induced HUS, several reports suggested pathological roles of JNK and p38 MAP kinase. For example, in human monocytes, Shiga toxin-1 induced phosphorylation of JNK and p38 MAP kinase, leading to production of TNF-α [12]. Shiga toxin also activated p38 MAP kinase in microvascular endothelial cells, which is responsible for cytotoxicity [13,14]. Furthermore, treatment of rats with an inhibitor of p38 MAP kinase significantly improved symptoms of toxin-induced HUS [15]. In contrast to stress-inducible, pro-apoptotic JNK and

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p38 MAP kinase, several previous reports identified ERK as a pro-survival kinase [16]. Currently, however, there is no report regarding involvement of ERK in the regulation of apoptosis in bacterial toxin-exposed cells. In the present investigation, we first examine whether and how ERK contributes to the regulation of apoptosis in SubAB-treated cells.

Like MAP kinase pathways, serine/threonine protein kinase Akt, also known as protein kinase B, mediates effects of extracellular signals on various cellular processes including growth, differentiation, survival and metabolism [17]. We recently reported that SubAB induces transient activation of Akt in renal tubular cells [18]. Because, in general, the Akt pathway delivers pro-survival signals [19], we next examine whether and how Akt is involved in the regulation of apoptosis in SubAB-exposed cells.

SubAB selectively cleaves GRP78 [6], causing ER stress and consequent unfolded protein response (UPR) [20]. ER stress activates three major branches of the UPR, and the protein kinase-like ER kinase (PERK)–eukaryotic translation initiation factor 2α (eIF2α) pathway is especially important in the survival of cells under ER stress conditions [21–25]. In the last part of this study, we examine roles of the PERK–eIF2α pathway in the regulation of ERK and Akt. Our current results suggest that SubAB triggers pro-survival pathways mediated by ERK and Akt and that the PERK–eIF2α branch of the UPR is responsible for the activation of these kinases.

2. Materials and methods

2.1. Reagents

SubAB and its inactive mutant $SubA_{A272}B$ were purified by Ni–NTA chromatography from recombinant *Escherichia coli*, as described before [3,26]. These agents were used at 10–20 ng/ml. Tunicamycin and thapsigargin were purchased from Sigma–Aldrich Japan (Tokyo, Japan). 4-Phenylbutyric acid (4-PBA), salubrinal, PD98059 and Akti-1/2 were obtained from Calbiochem (San Diego, CA).

2.2. Cell culture

The rat renal tubular epithelial cell line NRK-52E was purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (Gibco-BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS). Experiments were performed in the presence of 1% FBS.

2.3. Establishment of stable transfectants

NRK/PERKDN cells were established by stable transfection with a dominant-negative mutants of PERK. The expression plasmid pcDNA3-hPERK.K621M was provided by Dr. Ronald C. Wek (Indiana University School of Medicine) [27]. NRK-52E cells transfected with pcDNA3.1 (Invitrogen, Carlsbad, CA) were used as a control (NRK/Neo).

2.4. Northern blot analysis

Total RNA was extracted by a single-step method, and Northern blot analysis was performed as described before [28]. cDNAs for GRP78 (provided by Dr. Kazunori Imaizumi, University of Miyazaki) [29] and CCAAT/enhancer-binding protein-homologous protein (CHOP) [30] (provided by Dr. David Ron, New York University School of Medicine) were used to prepare radio-labeled probes. Expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control.

2.5. Western blot analysis

Western blot analysis of ERK and Akt was performed by the enhanced chemiluminescent system (Amersham Biosciences, Buckinghamshire, UK) using PhosphoPlus p44/42 MAP Kinase (Thr^{202}/Tyr^{204}) Antibody Kit and PhosphoPlus Akt (Ser473) Antibody Kit following protocols provided by Cell Signaling (Beverly, MA). Following antibodies were also used for Western blot analyses: anti-PERK (H-300) antibody, anti-phospho PERK (Thr981) antibody and anti-eIF2α antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho eIF2α antibody and anti-caspase-3 antibody from Cell Signaling; and anti-β-actin antibody from Sigma–Aldrich Japan.

2.6. Hoechst staining

Cells were fixed in 4% formaldehyde for 10 min and stained by Hoechst33258 (10 μg/ml; Sigma–Aldrich Japan) for 1 h. Because, in some situations, round cells were easily detached from the bottoms of culture plates, floating and attached cells were evaluated separately by fluorescence microscopy.

2.7. Trypan blue analysis

Cells were seeded in 24-well plates and treated with SubAB. Both attached cells and detached cells were harvested separately and subjected to trypan blue analysis. The percentages of dead cells against total cells were evaluated in individual wells. Assays were performed in quadruplicate.

2.8. Formazan assay

The number of viable cells was assessed by a formazan assay using Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan).

2.9. Statistical analysis

In the assessments of cell death, experiments were performed in quadruplicate. Data were expressed as means ± SE. Statistical analysis was performed using the non-parametric Mann–Whitney *U* test to compare data in different groups. A p value <0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Induction of apoptosis by SubAB

Previous reports suggested that SubAB induces apoptosis in Vero cells and HeLa cells [7,8,10]. We first examined apoptotic responses of NRK-52E cells to SubAB. Cells were treated with 1–25 ng/ml of SubAB, and microscopic analysis was performed. As shown in Supplementary Fig. S1A, SubAB induced cellular damage in a concentration-dependent manner. Hoechst staining exhibited nuclear condensation and fragmentation in SubAB-treated cells (Supplementary Fig. S1B). Western blot analysis showed modest activation of caspase-3 following exposure to 20 ng/ml SubAB (Supplementary Fig. S1C). These results suggest that SubAB induces apoptosis of NRK-52E cells.

3.2. Activation of ERK by SubAB and its contribution to cell survival

ERK is known as a putative anti-apoptotic kinase that is activated by a variety of external stimuli [16]. First, we examined whether or not SubAB can induce activation of ERK. For this purpose, cells were treated with SubAB and subjected to Western blot