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Involvement of the Nrf2-proteasome pathway in the endoplasmic reticulum stress response in pancreatic β-cells

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ABSTRACT

The ubiquitin-proteasome system plays a central role in protein quality control through endoplasmic reticulum (ER)-associated degradation (ERAD) of unfolded and misfolded proteins. NF-E2-related factor 2 (Nrf2) is a transcription factor that controls the expression of an array of phase II detoxification and antioxidant genes. Nrf2 signaling has additionally been shown to upregulate the expression of the proteasome catalytic subunits in several cell types. Here, we investigated the role of Nrf2 in tunicamycin-induced ER stress using a murine insulinoma β-cell line, βTC-6. shRNA-mediated silencing of Nrf2 expression in βTC-6 cells significantly increased tunicamycin-induced cytotoxicity, elevated the expression of the pro-apoptotic ER stress marker Chop10, and inhibited tunicamycin-inducible expression of the proteasomal catalytic subunits Psmb5 and Psmb6. The effects of 3*H*-1,2-dithiole-3-thione (D3T), a small molecule Nrf2 activator, on ER stress were also examined in βTC-6 cells. D3T pretreatment reduced tunicamycin cytotoxicity and attenuated the tunicamycin-inducible Chop10 and protein kinase RNA-activated-like ER kinase (Perk). The protective effect of D3T was shown to be associated with increased ERAD. D3T increased the expression of Psmb5 and Psmb6 and elevated chymotrypsin-like peptidase activity; proteasome inhibitor treatment blocked D3T effects on tunicamycin cytotoxicity and ER stress marker changes. Similarly, silencing of Nrf2 abolished the protective effect of D3T against ER stress. These results indicate that the Nrf2 pathway contributes to the ER stress response in pancreatic β-cells by enhancing proteasome-mediated ERAD.

Introduction

The endoplasmic reticulum (ER) is the organelle that biosynthesizes and modifies secretory proteins. The fidelity of protein synthesis in the ER is ensured by multiple protein quality control mechanisms and the multi-step processes of protein folding and maturation are tightly controlled to maintain homeostasis. However, stress conditions such as glucose starvation, oxidative stress, hypoxia, and high fat or cholesterol perturb protein homeostasis and lead to accumulation of misfolded proteins in the ER lumen, which is referred to as ER stress (Schroder and Kaufman, 2005). In response to ER stress, cells initiate a specialized buffering system known as the unfolded protein response (UPR), which activates cellular signaling pathways that restore protein homeostasis and ER function (Hampton, 2000; Schroder and Kaufman, 2005; Walter and Ron, 2011). The UPR is activated by 3 stress sensors in the ER: protein kinase RNA-activated-like ER kinase (Perk), inositol-requiring enzyme 1α (Ire1α), and activating transcription factor 6 (Atf6). Signaling pathways from these sensors reduce the ER load by attenuating mRNA translation, increasing mRNA degradation, and increasing the expression of molecular chaperones and components of the ER-associated degradation (ERAD) pathway (Tsai and Weissman, 2010, 2011). Overall, the UPR alleviates ER stress by enhancing its protein folding capacity and facilitating the removal of unfolded proteins. Although the UPR promotes cell survival, prolonged and severe ER stress can induce apoptosis (Shore et al., 2011; Woehlbier and Hetz, 2011). Thus, UPR sensors can also elevate expression of pro-apoptotic factors such as Chop10 (also known as growth arrest and DNA-damage-inducible 153, GADD153) and suppress anti-apoptotic factors such as Bcl-2.

The 26S proteasome, consisting of the 19S regulatory cap and the proteolytic 20S core complex, is responsible for the degradation of abnormal proteins in the cytosol, nucleus, and ER lumen (Poppek

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; ERAD, ER-associated degradation; iNrf2, NRF2 knockdown βTC-6; iSc, nonspecific control βTC-6; ARE, antioxidant-response element; GSH, glutathione; Nqo1, NAP(D)H, quinone oxidoreductase-1; Gclc, catalytic subunit of γ-glutamate cysteine ligase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; D3T, 3*H*-1,2-dithiole-3-thione.

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and Grune, 2006; Tai and Schuman, 2008; Xie, 2010). The core 20S proteasome is composed of several structural subunits as well as the catalytic subunits Psmb5, Psmb6, and Psmb7, which possess chymotrypsin-like, caspase-like, and trypsin-like peptidase activities, respectively (Bedford et al., 2010). The 19S proteasome controls access of substrate proteins to the inner part of the 20S proteasome by recognizing and degrading polyubiquitinated proteins in an ATP-dependent manner. The proteasome is one component of the UPR that mediates ERAD (Rubinsztein, 2006; Tai and Schuman, 2008; Xie, 2010). However, little is known about the molecular control of proteasome expression in mammalian cells. Previously, we demonstrated that the transcription factor NF-E2-related factor 2 (Nrf2) is involved in regulating the inducible expression of Psmb5 in murine cells (Kwak et al., 2003a). Nrf2 is an essential component of a transcriptional complex that activates expression of genes encoding detoxifying enzymes (e.g., NAD(P)H: quinone oxidoreductase 1 [Nqo1]) and antioxidant proteins (e.g., the GSH biosynthetic enzyme γ-glutamate cysteine ligase [Gcl]) (Li and Kong, 2009; Taguchi et al., 2011). Thus, the observation that Nrf2 upregulates Psmb5 has led to a novel concept that the proteasome can participate in the cellular defense system against divergent sources of stress.

Chronic ER stress and dysregulation of UPR have been proposed to contribute to various human diseases, including neurodegenerative diseases and disorders of pancreatic β-cell function (Back and Kaufman, 2012; Rubinsztein, 2006). Alzheimer's and Parkinson's diseases show common pathological lesions of accumulated abnormal protein aggregates. Consistent with this, changes in proteasome activity have been identified in experimental cell systems as well as in pathologic samples from patients with Alzheimer's or Parkinson's diseases (Mittal and Ganesh, 2010; Rubinsztein, 2006; Zabel et al., 2010). In particular, multiple lines of evidence suggest that ER stress is involved in the pathology of type 2 diabetes (Back and Kaufman, 2012; Fonseca et al., 2011). For example, chronic ER stress is increased in pancreatic cells from patients with diabetes, which can lead to β-cell death. In the present study, we have investigated the role of Nrf2 in tunicamycin-induced ER stress using a murine pancreatic β-cell line. Tunicamycin is a mixture of homologous nucleotide antibiotics that inhibit the synthesis of N-linked glycoproteins and is commonly used to induce ER stress experimentally. Our results show that 3*H*-1,2-dithiole-3-thione (D3T), a small molecule Nrf2 activator, attenuates tunicamycin-induced cytotoxicity and inhibits the increase in ER stress markers such as Chop10. We show that D3T treatment protects cells from tunicamycin-induced stress by increasing expression of proteasome catalytic subunits in an Nrf2-dependent manner. Consistent with this, Nrf2-silenced β-cells were more susceptible to tunicamycin cytotoxicity and this was not attenuated by D3T treatment.

Materials and methods

Materials. D3T was provided by Dr. Thomas Curphey (Dartmouth Medical School, NH). Tunicamycin was purchased from Sigma-Aldrich (Saint Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Amersco Inc. (Solo, OH, USA). MG132 and substrates for measurement of proteasome activity were from EMD Chemicals (Billerica, MA, USA). Primary antibodies for Western blotting of Psmb5 and Psmb6 were purchased from Enzo life sciences (Farmingdale, NJ, USA). Antibodies recognizing Nrf2 and β-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies for phospho-eIF2α and phosphor-Perk were obtained from Cell Signaling Technology (Beverly, MA, USA). The lentiviral expression plasmid for mouse Nrf2 short hairpin RNA (shRNA), Mission™ Lentiviral Packaging Mix, hexadimethrine bromide, and puromycin were all from Sigma-Aldrich.

Cell culture. The murine pancreatic β-insulinoma cell line βTC-6 was obtained from American Type Culture Collection (Manassas, VA, USA). βTC-6 cells were maintained in Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and penicillin/streptomycin (Hyclone). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Production of shRNA lentiviral particles. Lentiviral particles containing the Nrf2-specific shRNA or control, scrambled (Sc) shRNA were produced by transfection of HEK293T cells with the relevant shRNA expression plasmid and Mission™ Lentiviral Packaging Mix (Sigma-Aldrich) as described previously (Kim et al., 2011). Briefly, HEK293T cells were seeded in 60 mm plates at a density of 7×10^5 cells per well. The next day, the medium was replaced by OptiMEM (Invitrogen, Carlsbad, CA, USA) and cells were transfected with 1.5 μg pLKO.1-Nrf2 shRNA (mouse Nrf2-specific shRNA: 5′-CCGGCCAAAGCTAGTATAGCAA TAACTCGAGTTATTGCTATACTAGCTTTGGTTTTTG-3′) or pLKO.1-ScRNA and the Packaging Mix, using Lipofectamine™ 2000 (Invitrogen). On the second day, the medium was exchanged with fresh complete medium. The medium containing lentiviral particles was harvested after 4 days.

Stable transduction of βTC-6 cells. βTC-6 cells in 6-well plates were transduced with lentiviral particles containing either pLKO.1-ScRNA or pLKO.1-Nrf2 shRNA in the presence of 8 μg/ml of hexadimethrine bromide (Sigma-Aldrich). Transduction was continued for 48 h followed by 24 h recovery in complete medium. Stable plasmid-expressing cells were selected by growth for 4 weeks in medium containing 1 μg/ml puromycin (Sigma-Aldrich).

MTT assay. Cell viability was determined using the MTT assay. Briefly, βTC-6 cells were seeded at a density of 5×10^3 cells/well in 96-well plates and incubated with the relevant compounds (tunicamycin, D3T, MG132) for the indicated times. MTT solution (2 mg/ml) was added to the wells and plates were incubated for 4 h. The MTT solution was removed, 100 μl/well of DMSO was added, and the absorbance was measured at 540 nm using a Versamax microplate reader (Sunnyvale, CA, USA).

Total RNA extraction and RT-PCR analysis. Total RNA was isolated from βTC-6 cells using TRIzol reagent (Invitrogen). For the synthesis of cDNAs, reverse transcriptase (RT) reactions were performed by incubating 200 ng of total RNA with a reaction mixture containing 0.5 μg/μl oligo dT_{12-18} and 200 U/μl Moloney murine leukemia virus RT (Invitrogen). Quantitative real-time RT-PCR was performed using a Roche LightCycler (Mannheim, Germany) with the Takara SYBR Premix ExTaq system (Otsu, Japan). Primers were synthesized by Bioneer (Daejeon, South Korea) and the primer sequences for the mouse genes are shown in the online material. All mRNA levels were normalized to the level of Hprt transcripts.

Western blot analysis. βTC-6 cells were lysed with RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% NP40) containing a protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples were separated by electrophoresis on 6–12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany) using a Trans-Blot® Semi-Dry Cell (Bio-Rad). The membrane was then blocked with 3% skim milk for 1 h, and antibody incubations were performed. The chemiluminescent image was captured using a Fujifilm LAS-4000 mini imager (Fujifilm, Tokyo, Japan).

Measurement of luciferase activity. βTC-6 cells in 24-well plates were transfected with a mixture of 0.5 μg ARE-luciferase plasmid (Kim et al., 2011), 0.05 μg of pRLtk control plasmid (Promega, Madison, WI, USA), and WelFect transfection reagent (Welgene Inc., Daegu, South Korea). After 18 h, the transfection mixture was removed and cells