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Tunicamycin-induced unfolded protein response in the developing mouse brain

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ABSTRACT

Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) causes ER stress, resulting in the activation of the unfolded protein response (UPR). ER stress and UPR are associated with many neurodevelopmental and neurodegenerative disorders. The developing brain is particularly susceptible to environmental insults which may cause ER stress. We evaluated the UPR in the brain of postnatal mice. Tunicamycin, a commonly used ER stress inducer, was administered subcutaneously to mice of postnatal days (PDs) 4, 12 and 25. Tunicamycin caused UPR in the cerebral cortex, hippocampus and cerebellum of mice of PD4 and PD12, which was evident by the upregulation of ATF6, XBP1s, p-eIF2α, GRP78, GRP94 and MANF, but failed to induce UPR in the brain of PD25 mice. Tunicamycin-induced UPR in the liver was observed at all stages. In PD4 mice, tunicamycin-induced caspase-3 activation was observed in layer II of the parietal and optical cortex, CA1–CA3 and the subiculum of the hippocampus, the cerebellar external germinal layer and the superior/inferior colliculus. Tunicamycin-induced caspase-3 activation was also shown on PD12 but to a much lesser degree and mainly located in the dentate gyrus of the hippocampus, deep cerebellar nuclei and pons. Tunicamycin did not activate caspase-3 in the brain of PD25 mice and the liver of all stages. Similarly, immature cerebellar neurons were sensitive to tunicamycin-induced cell death in culture, but became resistant as they matured *in vitro*. These results suggest that the UPR is developmentally regulated and the immature brain is more susceptible to ER stress.

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Introduction

The endoplasmic reticulum (ER) is a subcellular organelle responsible for posttranslational protein processing and transport. Approximately one third of all cellular proteins are translocated into the lumen of the ER where posttranslational modification, folding and oligomerization occur. The ER is also the site for biosynthesis of steroids, cholesterol and other lipids. Cellular stress conditions, such as perturbed calcium homeostasis or redox status, elevated secretory protein synthesis rates, altered glycosylation levels and cholesterol overloading, can interfere with oxidative protein folding, leading to the accumulation of unfolded or misfolded proteins in the ER lumen. This causes ER stress and activates a compensatory mechanism, called the unfolded protein response (UPR) (Hetz et al., 2013; Wang and Kaufman, 2012). UPR attempts to relieve ER stress by two major pathways: the first is to halt the translation of unfolded proteins and enhance endoplasmic reticulum-associated degradation (ERAD) of unfolded or misfolded proteins; the second is to increase the expression of molecular chaperones to facilitate proper protein folding (Logue et al., 2013; Walter and Ron, 2011). However, when sustained or severe ER stress surpasses the capacity of UPR, apoptotic cell death occurs (Hetz et al., 2013; Logue et al., 2013).

ER stress and UPR participate in various physiological processes such as lipid and cholesterol metabolism, energy homeostasis, circadian function, cell surface signaling, development and cell differentiation (Hetz, 2012; Rutkowski and Hegde, 2010; Walter and Ron, 2011). ER

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CGNs, cerebellar granule neurons; DAB, 3,3′-diaminobenzidine; DAPI, 4,6-diamidino-phenylindole; DCN, deep cerebellar nucleus; DG, dentate gyrus; DIV, days *in vitro*; EGL, external germinal layer; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; FASD, fetal alcohol spectrum disorders; GFAP, glial fibrillary acidic protein; HD, Huntington's disease; IACUC, Institutional Animal Care and Use Committee; ICV, intracerebroventricular; IGL, internal granule layer; IP, intraperitoneal injection; MTT, 3-(4,5-dimethyl-thiazol-2yl)-2,5 diphenyltetrazolium bromide; NSCs, neural stem cells; PD, postnatal day; SC, subcutaneous; SUB, subiculum; UPR, unfolded protein response; WFS, Wolfram Syndrome.

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stress and UPR are also involved in many human diseases and disorders, such as inflammation, metabolic disorders, cardiovascular diseases, diabetes, obesity and cancer (Hetz et al., 2013; Wang and Kaufman, 2012; Yamamoto et al., 2010). ER stress has been shown to play an important role in the pathogenesis of various neurological diseases (Cornejo and Hetz, 2013; DeGracia and Montie, 2004; Endres and Reinhardt, 2013; Scheper and Hoozemans, 2009; Vidal et al., 2011; Xu and Zhu, 2012) and have been implicated in neurodegenerative processes in brain ischemia (Tajiri et al., 2004), Alzheimer's disease (AD) (Katayama et al., 2004), Parkinson's disease (PD) (Chen et al., 2004; Silva et al., 2005; Smith et al., 2005), Huntington's disease (HD) (Hirabayashi et al., 2001) and amyotrophic lateral sclerosis (ALS) (Turner and Atkin, 2006).

The developing brain is particularly susceptible to various environmental insults, *e.g.*, exposure to pollutants, infectious pathogens, heavy metals, drugs, alcohol, physical stress and malnutrition. These environmental factors often cause ER stress and induce UPR (Hettiarachchi et al., 2008; Ji, 2014; Kalinec et al., 2014; Ke et al., 2011; Kitamura, 2013; Oh et al., 2012; Pavlovsky et al., 2013; Qian and Tiffany-Castiglioni, 2003; Shin et al., 2007) and ER stress may account for some of their detrimental effects. However, the mechanisms underlying CNS damage caused by these environmental insults are complex; it may be mediated by the interplay of multiple factors, such as direct toxicity, oxidative stress or disruption of cellular metabolism. To evaluate the impact of ER stress on the developing brain, we need a model system which allows more specific induction of ER stress. Tunicamycin is an N-linked glycosylation inhibitor and is commonly used to induce ER stress experimentally. In this study, we evaluated tunicamycin-induced ER stress in the postnatal development of the mouse brain. We also studied tunicamycin-mediated neuroapoptosis.

Materials and methods

Materials. Tunicamycin and mouse anti-glial fibrillary acidic protein (GFAP) antibody were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-ATF6 antibody was purchased from LifeSpan Biosciences (Seattle, WA). Rabbit anti-Xbp1s antibody was purchased from Biolegend (San Diego, CA). Rabbit anti-p-eIF2α and cleaved caspase-3 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Rabbit anti-GRP78 antibody was obtained from Santa Cruz Biotechnology (Dallas, Texas). Rat anti-GRP94 antibody was obtained from Enzo Life Sciences (Farmingdale, NY 11735). Rabbit anti-MANF antibody was purchased from Abcam (Cambridge, MA). Mouse anti-neuronal nucleus (NeuN) antibody was obtained from Millipore Corporate (Billerica, MA). Mouse anti-tubulin, HRP-conjugated anti-rabbit, anti-mouse and anti-rat secondary antibodies were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Biotin-conjugated anti-rabbit secondary antibodies and ABC kit were obtained from Vector Laboratories (Burlingame, CA). Alexa-488 conjugated anti-rabbit and Alexa-594 conjugated anti-mouse antibodies were obtained from Life Technologies (Grand Island, NY). Ketamine/xylazine was obtained from Butler Schein Animal Health (Dublin, OH). Other chemicals and reagents were purchased either from Sigma Chemical or Life Technologies.

Animals and tunicamycin treatment. C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN) and maintained in the Division of Laboratory Animal Resources of the University of Kentucky Medical Center. All procedures were performed in accordance with the guidelines set by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky. Tunicamycin was administered on postnatal day 4 (PD4), PD12 and PD25. The mice received two subcutaneous injections of tunicamycin at 3 μg/g each and the injections were 2 h apart. Tunicamycin was diluted in 150 mM dextrose at a concentration of 0.3 μg/μl. The concentration was selected based on previous studies of tunicamycin injection in adult animals (Krokowski et al., 2013; Lee et al., 2012; Puthalakath et al., 2007; Rosenbaum et al., 2014; Sammeta and McClintock, 2010; Yamamoto et al., 2010). Mice in the control group were injected with the same amount of dextrose without tunicamycin. Mice were weighed at 0 and 24 h after the injection. Twenty four hours after the first injection, the mice were euthanized, and the brains and livers were dissected and processed for further analysis.

Tissue preparation and immunoblotting. Mice were anesthetized by an intraperitoneal (IP) injection of ketamine/xylazine (100 mg/kg/10 mg/kg) and the brain and liver were dissected and immediately frozen in liquid nitrogen and then stored in −80 °C. The protein was extracted and subjected to immunoblotting analysis as previously described (Ke et al., 2011). Briefly, tissues were homogenized in an ice cold lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.5% NP-40, 0.25% SDS, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. Homogenates were centrifuged at 20,000 *g* for 30 min at 4 °C and the supernatant fraction was collected. After determining protein concentration, aliquots of the protein samples (30 μg) were separated on a SDS-polyacrylamide gel by electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% BSA or 5% nonfat milk in 0.01 M PBS (pH 7.4) and 0.05% Tween-20 (TPBS) at room temperature for 1 h. Subsequently, the membranes were probed with primary antibodies overnight at 4 °C. After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase. The immune complexes were detected by the enhanced chemiluminescence substrate (PerkinElmer, Waltham, MA). In some cases, the blots were stripped and re-probed with an anti-tubulin antibody. The density of immunoblotting was quantified with the software of Quantity One (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry. The procedure for immunohistochemistry (IHC) has been previously described with some modifications (Ke et al., 2011). Briefly, animals were deeply anesthetized with intraperitoneal injection of ketamine/xylazine and then intracardially perfused with PBS followed by 4% paraformaldehyde in PBS (pH 7.4). The brain tissues were removed, post fixed in 4% paraformaldehyde for an additional 24 h and then transferred to 30% sucrose in PBS until the tissues sunk to the bottom. The tissues were frozen in OCT compound and sectioned at 40 μm in a sagittal plane using a freezing sliding microtome (Leica Microsystems, Wetzlar, Germany). Floating sections were incubated in 0.3% H_2O_2/30% methanol in PBS for 10 min. After washing with PBS, sections were mounted on slides and dried. The slides were then blocked with 5% goat serum and 0.5% TritonX-100 in PBS for 1 h at room temperature. After blocking, the slides were treated with a rabbit anti-cleaved caspase-3 antibody (1:8000) overnight at 4 °C. After washing with PBS, slides were incubated with biotin-conjugated goat anti-rabbit secondary antibody (1:1000) for 1 h at room temperature and followed by washing with PBS. Avidin–biotin–peroxidase complex was prepared according to the manufacturer's instructions. The slides were incubated in the complex for 1 h at room temperature. After rinsing, the slides were developed in 0.05% 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, Inc.) containing 0.003% H_2O_2 in PBS.

Immunofluorescent staining. The procedure for immunofluorescent staining has been previously described with some modifications (Wang et al., 2007). Briefly, mice were anesthetized and perfused as described above. The brain sections were prepared at 15–20 μm thickness, mounted and dried. After blocking with 5% goat serum and 0.5% TritonX-100 in PBS for 1 h at room temperature, the slides were incubated with a rabbit anti-cleaved caspase-3 antibody together with anti-NeuN, anti-Iba-1 or anti-GFAP overnight at 4 °C. After rinsing in PBS, the sections were incubated with Alexa Fluor 488-conjugated

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