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Thiamine deficiency induces endoplasmic reticulum stress and oxidative stress in human neurons derived from induced pluripotent stem cells

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ABSTRACT

Thiamine (vitamin B1) deficiency (TD) plays a major role in the etiology of Wernicke's encephalopathy (WE) which is a severe neurological disorder. TD induces selective neuronal cell death, neuroinflammation, endoplasmic reticulum (ER) stress and oxidative stress in the brain which are commonly observed in many aging-related neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and progressive supranuclear palsy (PSP). However, the underlying cellular and molecular mechanisms remain unclear. The progress in this line of research is hindered due to the lack of appropriate *in vitro* models. The neurons derived for the human induced pluripotent stem cells (hiPSCs) provide a relevant and powerful tool for the research in pharmaceutical and environmental neurotoxicity. In this study, we for the first time used human induced pluripotent stem cells (hiPSCs)-derived neurons (iCell neurons) to investigate the mechanisms of TD-induced neurodegeneration. We showed that TD caused a concentration- and duration-dependent death of iCell neurons. TD induced ER stress which was evident by the increase in ER stress markers, such as GRP78, XBP-1, CHOP, ATF-6, phosphorylated eIF2α, and cleaved caspase-12. TD also triggered oxidative stress which was shown by the increase in the expression 2,4-dinitrophenyl (DNP) and 4-hydroxynonenal (HNE). ER stress inhibitors (STF-083010 and salubrinal) and antioxidant *N*-acetyl cysteine (NAC) were effective in alleviating TD-induced death of iCell neurons, supporting the involvement of ER stress and oxidative stress. It establishes that the iCell neurons are a *novel* tool to investigate cellular and molecular mechanisms for TD-induced neurodegeneration.

1. Introduction

Thiamine (vitamin B1) deficiency (TD) causes Wernicke's encephalopathy (WE) which is a neuropsychiatric syndrome characterized by opthalmoplegia, ataxia and memory loss (Rao et al., 1996; Todd and Butterworth, 1999; Ke et al., 2003). Brain tissues isolated from WE patients display gliosis, vascular damage, and neuronal loss in specific regions, such as the mammillary bodies, inferior olive, and thalamus (Troncoso et al., 1981; Kril, 1996; Gibson et al., 1999; Pannunzio et al., 2000). However, the cellular and molecular mechanisms underlying TD-induced neuronal damage remain unclear. In experimental models, which include *in vitro* neuronal cultures and animal models, TD induces selective neuronal cell death, neuroinflammation, glial activation, endoplasmic reticulum (ER) stress and oxidative stress in the central nervous system (CNS). These features are commonly observed in many aging-related neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and progressive supranuclear palsy (PSP) (Ke and Gibson, 2004). Therefore, experimental TD models are useful not only for studying the mechanisms of WE but also for that of neurodegeneration in aging-associated degenerative diseases (Schwab et al., 1996; Gibson and Zhang, 2001; Park et al., 2001).

Up to date, *in vitro* neuronal models, mainly rodent primary neuron cultures, have made significant contributions to our understanding of the mechanisms of TD's effects on neurons because they have many advantages in investigating cellular and molecular events (Wang et al., 2007a; Lee et al., 2010; Cruz et al., 2012). However, these rodent-derived *in vitro* models may not be an appropriate reflection of

Abbreviations: AD, Alzheimer's disease; ATF6, activating transcription factor 6; CHOP, transcriptional factor C/EBP homologous protein; DNP, dinitrophenol; ER, endoplasmic reticulum; eIF2α, eukaryotic initiation factor 2α; HD, Huntington's disease; HNE, 4-hydroxynonenal; hiPSCs, human induced pluripotent stem cells; NAC, *N*-acetyl cysteine NAC; PD, Parkinson's disease; PERK, protein kinase R-like endoplasmic reticulum kinase; PSP, progressive supranuclear palsy; ROS, reactive oxygen species; TD, thiamine deficiency; UPR, unfolded protein response; XBP1, X-box binding protein-1.

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native human neurons and their physiological relevance is questionable. The discovery of human induced pluripotent stem cells (hiPSCs) has been revolutionizing the research in neurotoxicity because these cells can be differentiated into neurons, providing a more biologically relevant model. For example, the hiPSCs obtained from AD patients may offer a good model to investigate the interplay of genetics and environmental factors in the context of AD pathogenesis. The neurons derived from hiPSCs are therefore a *novel* and powerful tool to study the genetics, cellular/molecular biology of human neurons in response to environmental impacts or neurotoxins. In this study, we describe for the first time the use of hiPSCs-derived neurons (iCell neurons) as a new platform for studying the mechanisms of TD-induced neurodegeneration. The iCell neurons were differentiated and cryopreserved by Cellular Dynamics International (Madison, WI) and consist of a purified pan-neuronal population comprised predominantly of gamma aminoisobutyric acid (GABA)ergic and glutamatergic neurons (Whitemarsh et al., 2012). We show here that TD causes death of iCell neurons and induces ER stress and oxidative stress. The inhibition of ER stress or oxidative stress significantly reduces TD-induced cell death. This study establishes that the iCell neurons are a *novel* and useful tool to investigate cellular and molecular mechanisms for TD-induced CNS damage.

2. Materials and methods

2.1. Reagents

Poly-D-lysine hydrobromide, amprolium, and laminin were purchased from Sigma (St. Louis, MO, USA). Rabbit anti-p-eIF2α, anti-caspase-12, anti-ATF6, anti-dinitrophenol (DNP) antibodies were obtained from Cell signaling Technology (Danvers, MA). Rabbit anti-GRP78 antibody was obtained from Novus Biologicals (Littleton, CO). Mouse anti-CHOP antibody was obtained from Thermo Fisher Scientific (Rockford, IL). Rabbit anti-4-hydroxynonenal (HNE) antibody was obtained from lifespan BioSciences (Seattle, WA). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from GE Healthcare life Sciences (Piscataway, NJ). Calcein AM was obtained from Thermo Fisher Scientific (Waltham, MA).

2.2. Culture of human neurons derived from induced pluripotent stem cells

Human neurons differentiated from induced pluripotent stem (iPS) cells (iCell neurons) were purchased from Cellular Dynamics International (Madison, WI, USA) and stored in frozen vials containing at least 2.5 million cells. These cells display characteristics of primary forebrain neurons and are widely used in the scientific community for cellular, molecular and electrophysiological studies (Chai et al., 2012; Haythornthwaite et al., 2012; Whitemarsh et al., 2012; Xu et al., 2013; Dage et al., 2014). Cells were handled according to the instructions provided by the manufacturer. Briefly, iCell neurons were removed from liquid nitrogen storage and thawed for 3 min in a 37 °C water bath. Cells were gently transferred to a 50 ml tube and 1 ml of the maintenance medium (provided by the manufacturer) was added drop-wise. Cells were swirled gently to minimize osmotic shock and an additional 8 ml of medium was slowly added. Cells were plated at a density of 4×10^4/well on 96-well plates, 9×10^4/well on 24-well plate, or 2×10^5/dish on 60-mm cell culture dishes. Plates or cell culture dishes were pre-coated with poly-L-ornithine (PLO) (0.01%) followed by a layer of laminin (3.3 μg/ml) on top. Cells were cultured in the maintenance medium at 37 °C in 5% CO_2. The medium was changed after 24 h, and then every other day thereafter until the start of the experiment. For immunocytochemistry (IHC) studies, iCell neurons (8×10^4/chamber) were cultured on glass chamber slides pre-coated with POL/laminin.

2.3. Cytotoxicity assessment

Cell viability was determined by CCK-8 kit which measures the activity of mitochondrial dehydrogenase (Dojindo, MD, USA). Briefly, 10 μl of CCK-8 solution was added the cultures and incubated for 2–3 h. The activity of mitochondrial dehydrogenase was determined by the color product, formazan which was measured at an absorbance of 450 nm using a microplate reader. In addition, cell viability and morphology were also determined by calcein AM labeling. Calcein AM is able to cross the cell membrane and is metabolized in the cytosol to produce fluorescent calcein by cytosolic esterases. Dead cells are either unable to metabolize calcein AM or retain fluorescent calcein. The cultures were treated with 1 μM calcein AM for 30 min at 37 °C in 5% CO_2, and then examined under a fluorescent microscope.

2.4. Immunoblotting

The procedure for immunoblotting has been previously described (Ke and Gibson, 2004; Wang et al., 2007a). Briefly, aliquots of proteins (30 μg) were loaded into the lanes of a sodium dodecyl sulfate-polyacrylamide gel. The proteins were separated by electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in 0.01 M Tris-buffered saline (TBS) (pH 7.4) and 0.05% Tween-20 (TBST) at room temperature for 1 h. Then, the membranes were incubated with primary antibodies directed against target proteins overnight at 4 °C. The final dilutions for primary antibodies were: GRP78, 1:1,000; XBP-1, 1:1,000; p-eIF2α, 1:1,000; Chop, 1:500; caspase-12, 1:1,000; HNE, 1:1,000; DNP, 1:1,000; and actin, 1:5,000. After two quick washes in TBST, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (Amersham, Arlington, Heights, IL) diluted at 1:5000 in TBST containing 5% BSA for 1 h. The immunocomplexes were detected by the enhanced chemiluminescence method (Amersham). The density of immunoblotting was quantified with the software of ImageJ (version 1.48; National Institutes of Health, Bethesda, MD).

2.5. Statistical analysis

The data were expressed as mean ± SEM. Statistical significance was determined by the one-way ANOVA followed by Scheffé's post-doc test at a significance level of 5%. The analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

3. Results

3.1. TD induces death of iCell neurons

Amprolium is a thiamine antagonist and widely used *in vitro* and *in vivo* to induce thiamine deficiency (Wang et al., 2007a). We examined the effect of amprolium on the viability of iCell neurons by CCK-8 assay and calcein AM staining (Fig. 1). Amprolium induced a concentration- and duration-dependent decrease of the viability of iCell neurons. At 1 and 2 mM, amprolium significantly reduced cell viability by 16% and 40% respectively after 5 days of exposure, and by 36% and 46%, respectively following 7 days of exposure (Fig. 1A). Amprolium at the lower concentration, 0.5 mM, also reduced cell viability, but took a longer time; it decreased cell viability by 15% after 7 days of exposure. It is interesting to note that at 0.5 mM, 3–5 days of exposure slightly increased cell viability (Fig. 1A). We used calcein AM staining to further determine the cell viability and morphology. Amprolium not only reduced the number of viable cells but also caused the loss of neurites in the iCell neurons (Fig. 1B).