Neuroscience 144 (2007) 1045–1056

THIAMINE DEFICIENCY INDUCES ENDOPLASMIC RETICULUM STRESS IN NEURONS

X. WANG,[a] B. WANG,[a] Z. FAN,[a] X. SHI,[a] Z.-J. KE[a]* AND J. LUO[a,b]**

[a]*Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, PR China*

[b]*Department of Microbiology, Immunology and Cell Biology, West Virginia University School of Medicine, Robert C. Byrd Health Sciences Center, Morgantown, WV 26506, USA*

Abstract—Thiamine (vitamin B1) deficiency (TD) causes region selective neuronal loss in the brain; it has been used to model neurodegeneration that accompanies mild impairment of oxidative metabolism. The mechanisms for TD-induced neurodegeneration remain incompletely elucidated. Inhibition of protein glycosylation, perturbation of calcium homeostasis and reduction of disulfide bonds provoke the accumulation of unfolded proteins in the endoplasmic reticulum (ER), and cause ER stress. Recently, ER stress has been implicated in a number of neurodegenerative models. We demonstrated here that TD up-regulated several markers of ER stress, such as glucose-regulated protein (GRP) 78, growth arrest and DNA-damage inducible protein or C/EBP-homologus protein (GADD153/Chop), phosphorylation of eIF2α and cleavage of caspase-12 in the cerebellum and the thalamus of mice. Furthermore, ultrastructural analysis by electron microscopic study revealed an abnormality in ER structure. To establish an *in vitro* model of TD in neurons, we treated cultured cerebellar granule neurons (CGNs) with amprolium, a potent inhibitor of thiamine transport. Exposure to amprolium caused apoptosis and the generation of reactive oxygen species in CGNs. Similar to the observation *in vivo*, TD up-regulated markers for ER stress. Treatment of a selective inhibitor of caspase-12 significantly alleviated amprolium-induced death of CGNs. Thus, ER stress may play a role in TD-induced brain damage. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

*Corresponding author.
E-mail address: ZJKe@sibs.ac.cn (Z. J. Ke).
**Correspondence to: J. Luo, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 294 Taiyuan Road, Shanghai, 200031, PR China. Tel: +86-21-54920926; fax: +86-21-54920921.
E-mail address: JLuo@sibs.ac.cn (J. Luo).

Abbreviations: AD, Alzheimer's disease; ATF, activating transcription factor; CGNs, cerebellar granule neurons; CM-H_2DCFDA, 5-(And-6-)-chloromethyl-2′,7′-dichlorodi-hydrofluorescein diacetate acetyl ester; DAPI, 4,6-diamidino-2-phenylindole; DCF, 2,7-dichlorofluorescein; eIF2α, α subunit of the eukaryotic initiation factor 2; ER, endoplasmic reticulum; GADD153, growth arrest and DNA-damage inducible protein; GRP, glucose-regulated protein; IRE, inositol requiring; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer saline; PD, Parkinson's disease; PERK, pancreatic endoplasmic reticulum kinase; ROS, reactive oxygen species; SmTN, submedial thalamus nuclei; TD, thiamine deficiency; TD7, day 7 after thiamine deficiency; TPBS, 5% nonfat dry milk or 5% BSA in 0.01 M phosphate buffer saline (pH 7.4) and 0.05% Tween-20; UPR, unfolded protein response; WKS, Wernicke-Korsakoff syndrome.

Key words: cell death, cerebellum, neurodegeneration, nutrition, vitamin B1.

Thiamine (vitamin B1) deficiency (TD) induces regionally selective neuronal death in the brains of humans and animals; these regions include the thalamus, midbrain, brainstem and cerebellum (Victor et al., 1989; Baker et al., 1999). TD-induced neuronal loss accompanies a mild and chronic impairment of oxidative metabolism as well as inflammatory responses and glial activation (Ke and Gibson, 2004). Selective cell death, inflammation, glial activation and abnormalities in oxidative metabolism are common in many aging-related neurodegenerative diseases, such as Alzheimer's disease (AD) (Gibson and Zhang, 2001), Parkinson's disease (PD) (Schwab et al., 1996) and progressive supranuclear palsy (Park et al., 2001). The neurological disorder that is most clearly associated with TD in humans is Wernicke-Korsakoff syndrome (WKS), which is characterized by severe memory loss, cholinergic deficits and selective cell death in specific brain regions (Victor et al., 1989; Todd and Butterworth, 1999; Calingasan and Gibson, 2000; Ke et al., 2003). These features of the TD model make it amenable to investigate the cellular mechanisms of neurodegeneration.

The mechanisms for aging-related neurodegeneration remain incompletely understood. Recently, endoplasmic reticulum (ER) stress has been implicated in various neurodegenerative processes, such as brain ischemia (Tajiri et al., 2004; Hayashi et al., 2003, 2004), AD (Katayama et al., 2004), PD (Silva et al., 2005; Smith et al., 2005), Huntington's disease (HD) (Hirabayashi et al., 2001) and amyotrophic lateral sclerosis (Turner and Atkin, 2006). The ER is an important organelle involved in 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 the biosynthesis of steroids, cholesterol and other lipids. A number of 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. This can subsequently lead to the accumulation of unfolded or misfolded proteins in the ER lumen and activate compensatory mechanism, which has been referred to as ER stress response or unfolded protein response (UPR) (Kaufman, 1999; Ron, 2002; Shen et al., 2004; Xu et al., 2005). Several sensors of ER stress have been identified. These include pancreatic endoplasmic reticulum kinase (PERK), the kinase encoded by the inositol requiring (IRE)

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2006.10.008

1 gene and activating transcription factor (ATF) 6. PERK phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF2α), which attenuates the initiation of translation in response to ER stress. The activation of IRE1 and ATF6 signaling promotes the expression of ER-localized chaperones, such as glucose-regulated protein (GRP) 78 and GRP94, which facilitate the restoration of proper protein folding within the ER (Kaufman, 1999; Ron, 2002; Shen et al., 2004; Xu et al., 2005). These protective responses result in an overall decrease in translation, enhanced protein degradation and increased levels of ER chaperones, which consequently increase the protein folding capacity of the ER. However, sustained ER stress ultimately leads to the apoptotic death of the cell (Rutkowski and Kaufman, 2004; Xu et al., 2005).

The cause for TD-induced neuronal damage remains unclear. Several potential mechanisms have been proposed; these include mitochondrial dysfunction (Park et al., 2000; Singleton and Martin, 2001), impairment of oxidative metabolism (Ke and Gibson, 2004; Gibson et al., 2005) and acidosis (Hakim and Pappius, 1983; Pannunzio et al., 2000). Since oxidative stress may cause ER stress (Hayashi et al., 2003; Xue et al., 2005) and TD induces oxidative stress, we hypothesized that TD may cause ER stress which contributes to TD-induced brain damage. With both *in vivo* and *in vitro* approaches, the current study was designed to determine whether TD induced ER stress in neurons.

EXPERIMENTAL PROCEDURES

Animals and reagents

Sprague–Dawley rats and C57BL/6J mice were obtained from Shanghai Laboratory Animal Co. Ltd (Shanghai, China). The procedure for animal surgery was performed in accordance with the Guideline of Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS). Every effort was made to minimize the number of animals used and their suffering. 5-(And-6-)-chloromethyl-2′,7′-dichlorodi-hydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) was obtained from Molecular Probes (Eugene, OR, USA). A specific inhibitor for caspase-12 (Z-ATAD-FMK) was purchased from R&D Systems (cat. #:1079–100, Minneapolis, MN, USA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise mentioned. Anti-growth arrest and DNA-damage inducible protein (GADD153) antibody was obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-caspase-12 antibody was obtained from Calbiochem (La Jolla, CA, USA). Anti-α-tubulin antibody was purchased from Sigma Chemical Co. Anti-GRP78 antibody was obtained from StressGen Bioreagents (Ann Arbor, MI, USA). All other antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cultures of cerebellar granule neurons (CGNs)

Thirty-six- or seven-day-old Sprague–Dawley rat pups were used in the study. Cultures of CGNs were generated using a previously described method with a slight modification (Chen et al., 2004). Briefly, the rat pups were decapitated under deep anesthesia and cerebella removed. The cerebella were minced with a sterile razor blade and suspended in 10 ml of trypsin solution (0.025%) at 37 °C. After incubation for 15 min, an equal volume of a solution containing DNAse (130 Kunitz units/ml) and trypsin inhibitor (0.75 mg/ml) was added, and the tissue was sedimented by a brief (5 s) centrifugation. The tissue was dissociated by trituration, and the cell suspension was mixed with 4% bovine serum albumin and centrifuged. The cell pellet was re-suspended in Neurobasal/B27 medium containing B27 (2%), KCl (25 mM), glutamine (1 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were plated into poly-D-lysine (50 μg/ml) -coated cell culture wells or dishes, and maintained at 37 °C in a humidified environment containing 5% CO_2.

Induction of TD

The animal TD model has been previously described (Ke et al., 2003). Briefly, adult male C57BL/6J mice (20–25 g) were housed in a controlled environment (one mouse/cage at 23 °C and 53% humidity). The animals were fed with either a control diet or a thiamine deficient diet (ICN Nutrition Biomedicals, Cleveland, OH, USA) *ad libitum*. TD animals also received a daily i.p. injection of a thiamine antagonist, pyrithiamine hydrobromide (5 μg/10 g body weight, Sigma Chemical Co.), while control animals were injected with saline. Pyrithiamine is a potent inhibitor of thiamine pyrophosphokinase and blocks the synthesis of thiamine diphosphate (TDP). A total of 120 animals were used for immunoblotting analysis. There were 15 animals per treatment group and the brains from these animals were combined to generate three independent pooled tissue samples (five animals per pooled sample, $n=3$). The brain tissues were combined to minimize the variation among individual animals. The statistical analyses (one-way ANOVA) were carried out on samples from three independent pools of brains.

TD in CGNs was induced by the treatment of amprolium. Amprolium is a competitive inhibitor of thiamine transport and effectively depletes intracellular thiamine (Bettendorff et al., 1995; Park et al., 2000). After cultured in Neurobasal/B27 medium for 7 days, CGNs were treated with amprolium (0, 0.5, 1 or 1.5 mM) for indicated times.

Sample preparation

After treatment, the mice were killed by decapitation and the cerebella and thalamus were immediately dissected from TD and control mice. Animals were anesthetized by i.p. injection of chloral hydrate (350 mg/kg). The thalamus was dissected according to the method of Glowinski and Iversen (1966). The tissues were frozen in liquid nitrogen and stored at −80 °C for later analysis. Proteins were extracted with a previously described method (Li et al., 2002). 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 20,800×g for 30 min at 4 °C and the supernatant fraction was collected.

For protein extraction in cultured neurons, the CGNs were lysed in an ice-cold lysis buffer that contained 5 mM EDTA, 1% NP-40, 10 mg/ml PMSF, 10 μg/ml leupeptin and 100 mM sodium orthovanadate in phosphate buffer saline (PBS), and centrifuged at 20,800×g for 10 min. The supernatant was designated as the cytoplasmic fraction. The pellets were sonicated in a nuclear extraction buffer (20 mM Tris–HCl, pH 7.5, 1% SDS, 5 mM EGTA, 0.5% Triton X-100, 150 mM NaCl, 1 mM DTT, 10 μg/ml leupeptin and 1 mM Pephabloc s.c.) and centrifuged at 20,800×g for 10 min. The supernatant was collected and designated as the nuclear fraction.

Immunoblotting

The procedure for immunoblotting has been previously described (Li et al., 2002). Aliquots of cytoplasmic or nuclear proteins (50 μg)