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Altered expression of tight junction proteins and matrix metalloproteinases in thiamine-deficient mouse brain

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ABSTRACT

Wernicke's encephalopathy (WE) in humans is a metabolic disorder caused by thiamine deficiency (TD). In both humans and experimental animals, TD leads to selective neuronal cell death in diencephalic and brainstem structures. Neuropathologic features of WE include petechial hemorrhagic lesions, and blood–brain barrier (BBB) breakdown has been suggested to play an important role in the pathogenesis of TD. The goal of the present study was to examine expression of the tight junction (TJ) protein occludin, its associated scaffolding proteins zona occludens (ZO-1 and ZO-2), and to measure matrix metalloproteinase (MMP) levels as a function of regional BBB permeability changes in thiamine-deficient mice. TD was induced in 12-week-old male C57Bl/6 mice by feeding a thiamine-deficient diet and administration of the central thiamine antagonist pyrithiamine. BBB permeability was measured by IgG extravasation; expression of occludin, ZO-1 and ZO-2 was measured by Western blot analysis and RT-PCR, structural integrity of the BBB was assessed using occludin and ZO-1 immunostaining, and MMPs levels were measured by gelatin zymography and immunohistochemistry. Studies were performed in vulnerable (medial thalamus) versus spared (frontal cortex) regions of the brain. Hemorrhagic lesions, selective increases in brain IgG extravasation, a concomitant loss in protein expression of occludin, ZO-1 and ZO-2, as well as decreased and disrupted patterns of occludin and ZO-1 immunostaining were observed in the medial thalamus of thiamine-deficient mice. MMP-9 levels were also selectively increased in the medial thalamus of these animals, and were found to be localized in the vascular endothelium, as well as in cells with an apparent polymorphonuclear morphology. No changes of TJ gene expression were observed. These results indicate that alterations in TJ proteins occur in TD, and offer a plausible explanation for the selective increase in BBB permeability in thiamine-deficient animals. They also suggest a role for MMP-9 in the initiation of changes to BBB integrity in TD.

1. Introduction

Wernicke's encephalopathy (WE) in humans is a metabolic disorder caused by thiamine deficiency (TD) and is characterized clinically by ophthalmoplegia, ataxia and confusion. Neuropathologic features of WE include selective neuronal cell loss and petechial hemorrhagic lesions in mamillary bodies, thalamus and cerebellum with relative sparring of the other brain structures. Given the hemorrhagic nature of the lesions, blood–brain barrier (BBB) breakdown is considered to play an important role in the pathogenesis of WE. Animal models of TD recapitulate the neuropathologic features of WE (Troncoso et al., 1981; Heroux and Butterworth, 1992), and BBB disruption has been shown to occur using classical techniques in these models (Harata and Iwasaki, 1995; Calingasan et al., 1995; Todd and Butterworth, 1999). Furthermore, it has been suggested that dysfunction of the cerebrovascular endothelium predicts regional vulnerability to TD (Calingasan and Gibson, 2000; Kruse et al., 2004).

The BBB is a highly specialized structure composed of a tightly sealed monolayer of cerebrovascular endothelial cells connected at a junctional complex by tight junctions (TJs) (Hawkins and Davis, 2005). The BBB/TJ network comprises integral membrane proteins such as occludin linked to the cytoplasmic scaffolding zona occludens proteins (ZO-1, ZO-2). Expression of these proteins is frequently modified in a wide range of neurological disorders including multiple sclerosis, stroke and Alzheimer's disease (Zlokovic, 2008). Matrix metalloproteinase (MMPs), also known as gelatinases, are zinc-dependent endopeptidases that specialize in the degradation of components of the extracellular matrix and basement membrane of the cell, and are implicated in many brain

Abbreviations: WE, Wernicke's encephalopathy; TD, thiamine deficiency; BBB, blood–brain barrier; ZO, zona occluden; TJ, tight junction; MMP, matrix metalloproteinase; NO, nitric oxide; eNOS, endothelial nitric oxide synthase.

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disorders associated with BBB breakdown such as cerebral ischemia (Rosenberg et al., 1998) and experimental multiple sclerosis (Kieseier et al., 1999). However, no studies to date have addressed the possible role of TJ proteins and MMPs in the BBB changes associated with TD.

The goal of the present study therefore was to examine expression of the common TJ protein occludin, its associated scaffolding proteins ZO-1 and ZO-2, as well as the related MMPs (MMP-2, MMP-9) as a function of regional BBB permeability changes due to TD. Studies were conducted at the symptomatic stage of TD, characterized by hemorrhagic lesions, and evaluations were made in a vulnerable brain structure (medial thalamus) compared to a relatively spared brain structure (frontal cortex). Use was made of a well characterized mouse model of TD previously validated in several laboratories (Ke and Gibson, 2004; Watanabe et al., 1981).

2. Experimental procedures

2.1. Animal procedures

12-week-old male C57Bl6 mice (Charles River, St-Constant, QC) were used for all experiments. Mice were housed individually under constant conditions of temperature, humidity and 12 h light/dark cycles and had free access to water at all times. Mice were allowed to adapt to their environment for at least 3 days prior the initiation of treatments and were randomly assigned to either TD or pair-fed control (PFC) groups. All experimental procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital (CHUM) and the University of Montreal.

2.2. Thiamine deficiency protocol

Mice in the TD group ($n = 8$) were fed a thiamine-deficient diet (ICN Nutritional Biochemicals, Cleveland, OH) and administered daily pyrithiamine hydrobromide (Sigma–Aldrich, St Louis, MO) (0.5 mg per kg body weight) intraperitoneally. Control mice ($n = 8$) were pair-fed to equal food consumption with the thiamine-deficient mice using the same thiamine-deficient diet and supplemented with daily i.p. injections of thiamine (0.1 mg per kg body weight). Treatments were continued until mice displayed loss of righting reflex (the animal is no longer able to right itself when placed on its back). For the purpose of the present study, this stage was defined as the symptomatic stage of encephalopathy. Any mice exhibiting spontaneous seizures were eliminated from the protocols. Animals were sacrificed by decapitation, the brains were promptly removed and flash frozen in isopentane on dry ice and stored at −80 °C. They were then dissected on ice into frontal cortex and medial thalamus, according to the mouse brain atlas of Paxinos and Franklin (2003).

2.3. Histopathology and immunohistochemistry

A second group of thiamine-deficient and PFC mice was used for histopathologic and immunohistochemical studies. These mice were anaesthetized with a ketamine/xylazine cocktail (50 mg/kg and 9 mg/kg, respectively) and perfused transcardially with 50 ml of saline followed by 100 ml of neutral-buffered formalin containing 4% formaldehyde, 0.5% sodium phosphate buffer, 1.5% methanol and 0.02% glutaraldehyde, pH 7.0 (Fisher Scientific, Fair Lawn, NJ). Brains were then removed and post-fixed 24 h in the same solution. Free-floating 50 μm coronal sections were cut using a sectioning vibratome at the level of the frontal cortex and the medial thalamus. Sections were mounted on slides, stained with cresyl violet 0.1% and mounted for examination. Neuronal cell numbers were assessed by counting four adjacent grid areas (0.4 mm^2) of cresyl violet-stained sections. Histological criteria for neuronal counting included nuclear size and the presence of a nucleolus.

For occludin, ZO-1 and MMP-9 immunohistochemistry, frontal cortex and medial thalamus sections were mounted on slides. For occludin and ZO-1 antigen retrieval, sections were incubated in a solution of protease type XIV at 37 °C for 15 min (2 mg/ml, Sigma–Aldrich), and for MMP-9, sections were boiled in a microwave for 10 min in 10 mM citrate buffer pH 6.0. Endogenous peroxidase activity was blocked in sections by means of 0.3% H_2O_2 in methanol for 15 min at room temperature and then washed with PBS. The non-specific binding sites were blocked using 10% normal goat serum or rabbit serum in 0.2% Triton X-100/PBS for 30 min. The sections were then incubated overnight with rabbit anti-occludin (1 μg/ml, Invitrogen, Carlsbad, CA), anti-ZO-1 (3 μg/ml, Invitrogen) and goat anti-MMP-9 (pro and active forms, 0.5 μg/ml, R&D systems, Minneapolis, MN) polyclonal antibodies at 4 °C. After washing in PBS, the sections were incubated with biotinylated goat anti-rabbit or rabbit anti-goat IgG secondary antibody (1:200, Vector Laboratories, Burlingame, CA), followed by incubation with ABC reagent (Vector Laboratories). Immunoreactivity was subsequently detected by incubation with 3,3′-diaminobenzidine containing urea hydrogen peroxide (Sigma–Aldrich). Sections were dehydrated in ethanol, cleared with xylene and processed for examination. Sections without primary antibodies were used as negative controls and showed no immunoreactivity.

2.4. Protein extraction

Medial thalamus and frontal cortex samples were homogenized at 4 °C in 50 mM Tris–HCl buffer, pH 7.4, containing a protease inhibitor cocktail (Sigma–Aldrich) using a Potter–Elvehjem tissue homogenizer. After centrifugation at 12,000 × g for 45 min, membranes were separated from the cytosolic fraction, washed in the same buffer and stored at −80 °C. Protein concentrations were estimated using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard.

2.5. Western blotting

Membrane fractions from medial thalamus and frontal cortex (10–60 μg protein equivalent) were solubilized in Laemmli buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 0.1% bromophenol blue) and boiled for 5 min. Proteins were resolved on 7–9% denaturing SDS–polyacrylamide gels and transferred overnight at 4 °C to PVDF membranes (Bio-Rad Laboratories). Membranes were blocked by incubation for 1 h at room temperature in Tris buffered saline containing 0.05% Tween 20 (TBST) and 5% dry milk, then incubated for 1 h with rabbit polyclonal antibodies directed against occludin (1 μg/ml), ZO-1 (0.5 μg/ml), ZO-2 (1 μg/ml), (Invitrogen) and mouse monoclonal antibody directed against β-actin (1/100,000) (Sigma–Aldrich). Membranes were washed several times with TBST and incubated for 1 h with an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Perkin-Elmer Life Sciences, Boston, MA, USA). After extensive washing with TBST, peroxidase activity was detected by enhanced chemiluminescence using the ECL detection system (Amersham, Arlington Heights, IL, USA). Intensity of the bands was measured by densitometry and quantified using Quantity-One software (Bio-Rad Laboratories). Results are expressed as percentage of the housekeeping protein β-actin to normalize for loading variations.

2.6. IgG extravasation

Ten μg of cytosolic fraction from samples of medial thalamus and frontal cortex were solubilized in Laemmli buffer and boiled for 5 min. Proteins were resolved on 9% denaturing SDS–polyacrylamide gels and transferred overnight at 4 °C to PVDF membranes. Membranes were blocked by incubation for 1 h at room temperature in Tris buffered saline containing 0.05% Tween 20 (TBST) and 5% dry milk then incubated for 1 h with an anti-mouse-horseradish peroxidase-conjugated secondary antibody (1:3000, Perkin-Elmer Life Sciences). After extensive washing with TBST, peroxidase activity was detected by enhanced chemiluminescence using the ECL detection system (Amersham). Intensity of the bands (IgG light chain) was measured by densitometry using Quantity One software (Bio-Rad Laboratories). The housekeeping protein β-actin was used to normalize for loading variations.

2.7. SDS–PAGE gelatin zymography

This assay detects both MMP-9 and MMP-2 gelatinases. Twenty μg of sample (cytosolic fraction) was electrophoresed at 4 °C in 10% SDS–PAGE containing 1 mg/ml of gelatin (Bio-Rad Laboratories). Mixtures (1.5 ng) of recombinant MMP-9 and MMP-2 (US Biological, Cleveland, OH) were used as standards. The gels were processed, incubated in 2.5% Triton X-100 for 1 h, incubated in a zymogen buffer (50 mM Tris, pH 7.4, 5 mM $CaCl_2$, 200 mM NaCl, 0.02% Brij.35) at 37 °C for 40 h, stained with 0.5% Coomassie Blue R-250 for 3 h, and destained with three changes of 30% methanol, 10% acetic acid (for 15, 30, and 60 min of destain time, respectively, for each change). Finally gels were incubated for 15 min in 30% methanol, 5% glycerol prior to drying between sheets of cellophane. Dried gels were scanned and the bands of activity were quantified using Quantity One software (Bio-Rad laboratories). Gelatinase activity presents as a clear band against a blue background.

2.8. RNA extraction

Total RNA was extracted from medial thalamus using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Putative contaminating DNA was eliminated by adding 100 U of RNase-free DNase I per 50 μg of total RNA at 37 °C for 1 h. Purified RNA was then extracted with phenol, precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water. RNA samples were kept at −80 °C until use.

2.9. Semi-quantitative RT-PCR analysis

Expression of ZO-1, ZO-2 and occludin was investigated by one-step semi-quantitative reverse transcription-polymerase chain reaction. 18S rRNA was used as an internal standard to monitor loading variations. Total RNA (1 μg) was mixed with 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) bovine