Research report

Dietary restriction attenuates the neuronal loss, induction of heme oxygenase-1 and blood–brain barrier breakdown induced by impaired oxidative metabolism

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Abstract

Experimental thiamine deficiency (TD) is a model of impaired oxidative metabolism associated with region-selective neuronal loss in the brain. Oxidative stress is a prominent feature of TD neuropathology, as evidenced by the accumulation of heme oxygenase-1 (HO-1), ferritin, reactive iron and superoxide dismutase in microglia, nitrotyrosine and 4-hydroxynonenal in neurons, as well as induction of endothelial nitric oxide synthase within the vulnerable areas. Dietary restriction (DR) reduces oxidative stress in several organ systems including the brain. DR increases lifespan and reduces neurodegeneration in a variety of models of neuronal injury. The possibility that DR can protect vulnerable neurons against TD-induced oxidative insults has not been tested. The current studies tested whether approximately 3 months of DR (60% of ad libitum intake) altered the response to TD. Six month-old ad libitum-fed or dietary restricted C57BL/6 mice received a thiamine-deficient diet either ad libitum, or under a DR regimen respectively for eleven days. The TD mice also received daily injections of the thiamine antagonist pyrithiamine. Control ad libitum-fed or DR mice received an unlimited amount, or 60% of ad libitum intake, respectively, of thiamine-supplemented diet. As in past studies, TD produced region-selective neuronal loss (−60%), HO-1 induction, and IgG extravasation in the thalamus of ad libitum-fed mice. DR attenuated the TD-induced neuronal loss (−30%), HO-1 induction and IgG extravasation in the thalamus. These studies suggest that oxidative damage is critical to the pathogenesis of TD, and that DR modulates the extent of free radical damage in the brain. Thus, TD is an important model for studying the relationship between aging, oxidative stress and nutrition.

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1. Introduction

Experimental thiamine deficiency (TD) models the molecular and cellular mechanisms by which chronic aberrations in oxidative metabolism associated with well-defined biochemical lesions (i.e., reduction of thiamine-dependent enzyme activities) lead to selective neurodegeneration in brain. Reductions in thiamine-dependent enzymes also occur in many neurodegenerative disorders including Alzheimer’s disease [17], Parkinson’s disease [28], and Wernicke–Korsakoff syndrome [5]. As in these human diseases, oxidative stress is a prominent feature of TD-induced neurodegeneration in animals. TD induces indicators of oxidative stress including heme oxygenase-1, (HO-1) [10], superoxide dismutase [33], ferritin, and reactive iron [9] in microglia. TD also increases the nitration product of peroxynitrite, nitrotyrosine [9] and the lipid peroxidation product, 4-hydroxynonenal [10] in neurons within the vulnerable areas. Our recent studies suggest that early stages of TD induce intercellular adhesion molecule-1 (ICAM-1) and endothelial nitric oxide synthase (eNOS), indicating that oxidative stress to microvessels in the thalamus is a critical initial event in the pathogenesis of TD [8]. Furthermore, TD elevates the
concentration of reactive oxygen species in the thalamus [22]. Thus, although the mechanism underlying the region-specific neurodegeneration during TD is unknown, evidence for the role of oxidative stress is mounting.

 Dietary restriction (DR) is a well-established means of prolonging the lifespan of mammals [3,34]. DR may act by modulating or reducing oxidative stress in several organ systems [20]. This hypothesis is further strengthened by recent investigations suggesting that DR increases the resistance of neurons to a variety of oxidative insults. For example, DR protects neurons against MPTP-induced toxicity [14], excitotoxicity, and metabolic injury [4]. DR also protects hippocampal neurons in mice against the deleterious presenilin-1 mutation that is linked to early onset of Alzheimer’s disease [36].

 If increased oxidative damage is a key underlying mechanism for the sensitivity of selective neurons and the blood–brain barrier to TD, then it would be predicted that the dietary restricted, thiamine-deficient mice would be less sensitive than the ad libitum fed, thiamine-deficient group. The current studies tested whether DR affords protection against selective neurodegeneration and blood–brain barrier breakdown during TD.

2. Materials and methods

2.1. Animals

Six month-old dietary restricted or ad libitum fed C57BL/6NNia mice were obtained from Harlan Sprague–Dawley (Indianapolis, IN) through the National Institute on Aging. C57BL/6NNia mice were used because the TD pathology in this strain has been extensively studied in our previous reports [7–10], and the DR paradigm has also been characterized in this strain [11]. A stepwise increase in DR regimen was implemented at the Harlan Sprague–Dawley facility, from 90% of the ad libitum intake at 12 0.1 ml of saline/10 g body weight; Sigma, St. Louis, MO). Dietary restricted controls (DR/C; n=5) received a thiamine-supplemented diet and intraperitoneal saline injection (0.1 ml/10 g body weight). The thiamine-deficient and thiamine-supplemented diets for the DR/TD and DR/C groups, respectively, were fed at 60% of the ad libitum intake. For the ad libitum group, TD was induced in 5 animals (AL/TD) as in the DR group except that the thiamine-deficient diet was not restricted. Ad libitum controls (AL/C; n=5) received an unlimited amount of thiamine-supplemented diet, and intraperitoneal saline injection.

2.3. Tissue preparation and immunocytochemistry

Mice were euthanized with halothane after 11 days of TD, and perfused transcardially with 0.9% NaCl solution followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4). Brains were removed and sectioned (35 μm thick).

Free-floating sections to be analyzed for neuronal loss were immunostained with an antibody against an excellent neuronal marker, neuron-specific nuclear protein, NeuN [29]. This method eliminates the problem of distinguishing between small interneurons and glial cells, which is encountered in routine hematoxylin-and-eosin or cresyl violet staining. As in our previous studies [8,10], sections were incubated in 0.05 M potassium phosphate buffered saline (KPBS) containing 1% NaOH and 3% H2O2 for 30 min. After rinsing in KPBS 3 times for 10 min each, sections were treated with 0.4% Triton X-100 and 1% bovine serum albumin (BSA) in KPBS for 30 min. The sections were incubated in NeuN antiserum (Chemicon, Temecula, CA; 1:1000 in KPBS/1%BSA/0.4%Triton) for 18 h. After rinsing in KPBS containing 0.25% BSA and 0.02% Triton X-100, sections were incubated in biotinylated anti-mouse IgG (1:200 in KPBS/0.25% BSA/0.02% Triton; Vector Laboratories, Burlingame, CA) for 1 h, followed by avidin–biotin–peroxidase complex (1:200 in KPBS; Vector) for 1 h. After rinsing in 0.05 M KPBS, the reaction was developed in 0.05% DAB and 0.003% H2O2 in KPBS and stopped with 3 washes of KPBS.

NeuN-stained sections were also used for the assessment of the blood–brain barrier integrity. Since the monoclonal NeuN antibody requires the use of anti-mouse IgG as secondary antibody, extravasation of IgG, a measure of blood–brain barrier breakdown [6,32] was immunohistochromically detectable in the same sections stained for NeuN. The pattern of IgG immunoreactivity in NeuN-stained sections was identical to that of semidistant sections that were stained specifically for mouse IgG following the standard method for detection of blood–brain barrier disturbances for IgG [32]. IgG immunoreactivity did not interfere with neuronal labeling with NeuN antibody.

Immunostaining for HO-1 employed a modified avidin–biotin–peroxidase immunocytochemistry [18] as described previously [8,10]. Sections were pretreated with 3% H2O2...
in 0.1 M sodium phosphate buffered saline (PBS) for 30 min. The sections were incubated sequentially in (a) 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS for 30 min, (b) HO-1 antibody (1:4000; StressGen Biotechnologies Corp., British Columbia, Canada) in 0.1 M PBS/0.5% BSA for 18 h, (c) biotinylated anti-rabbit IgG (Vector) diluted 1:200 in PBS/0.5% BSA for 1 h, and (d) avidin–biotin–peroxidase complex (Vector) diluted 1:200 in PBS for 1 h. The chromogen used was 0.05% 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB; Sigma) containing 0.003% H₂O₂ in PB.

The specificity of HO-1 antibody binding was confirmed by preadsorption experiments. HO-1 antiserum was incubated with 20 μg/ml purified HO-1 protein (StressGen) for 3 h at 37°C. Adjacent sections from TD brains were immunostained with the preadsorbed antiserum in parallel with the non-preadsorbed antibody. Methodological control experiments consisted of incubating the sections in PBS without the primary antibody.

2.4. Quantitative analysis of neuronal loss

Areal density nuclear profile counts were determined as indicators of neuronal cell numbers following the criteria of Coggleshall and Lekan [12]. As described in our previous studies [8,10], this strategy was used for several reasons: (a) Estimates from nuclear profile counts deviate less from true numbers compared to total profile counts which include cytoplasmic edges, since the diameter of the neuronal nuclei in the submedial thalamic nucleus is small; (b) TD did not alter the size of individual neuronal nuclei in the submedial nucleus; and (c) TD did not alter the overall size of the submedial nucleus.

NeuN-immunostained sections were used for quantifying neuronal loss by an investigator who had no knowledge of the feeding regimen or the experimental treatment. Neuronal counts were obtained for an area covering the submedial thalamic nucleus (previously called gelatinosus nucleus) where TD-induced neuronal loss is first detected within the thalamus in mice [10], as in rats [35]. Using the mouse brain atlas of Franklin and Paxinos [16] as a guide, sections through three rostrocaudal levels (175 μm apart) of the submedial thalamic nucleus were analyzed under the microscope. Total neuronal cell counts were made within a 0.46 mm² area for each side of the brain. This area encompasses the submedial nucleus and part of adjacent subnuclei. Care was taken to position a calibrated eyepiece grid by using the mammillothalamic tract as a landmark. Results are presented as means of three total counts from different rostrocaudal levels.

2.5. Quantitative analysis of HO-1 induction

For evaluation of HO-1 induction, HO-1-stained sections adjacent to those used for NeuN immunoreactivity were used. HO-1 induction was measured by counting the intensely stained glial cells throughout the brain sections. For each animal, three sections 175 μm apart were examined. Only the intensely stained cells that were distinctly above the background (constitutive) level of staining were counted.

2.6. Statistical analysis

All values for neuronal and HO-1-labeled cell counts are expressed as means ± standard error of the mean (S.E.M.). Statistical significance of group differences was tested by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test.

3. Results

3.1. Behavioral characteristics

Before the initiation of TD induction, the body weight (g) of the DR mice was 19.6 ± 2 while that of the AL mice was 25.9 ± 4. TD normally reduces food intake, as evidenced by body weight loss. TD mice began to lose weight after 8 (n = 3 out of 5) or 9 (2 out of 5) days of TD. The difference between the body weight change of the TD ad lib group and the dietary restricted group was not statistically significant on days 8 (P = 0.857) and 9 (P = 0.335), but was significant on days 10 (P = 0.016) and 11 (P = 0.004). The AL/TD mice showed motor deficits by day 10 (n = 5/5) while the DR/TD mice exhibited these deficits on day 11 (n = 5/5).

3.2. Macroscopic neuropathological lesions

No apparent macroscopic differences were observed between the brains of AL/C and DR/C mice. As in our past studies [7–10], the brains of AL/TD mice exhibited pinpoint hemorrhages in the thalamus, mammillary body, inferior colliculus, dorsal lateral and medial geniculate nucleus, superior and inferior olives and some periventricular regions. The lateral ventricles showed edematous enlargement. Our previous work revealed that the submedial thalamic nucleus is the initial site of neuronal loss, which spreads to other nuclei until the whole thalamus is affected [8,10]. This consistent pattern makes it convenient to study the temporal relationships between neuronal loss and the region-selective neuropathological changes. Thus, the current studies focused on the thalamus, particularly the submedial nucleus.

3.3. Neuronal loss in thiamine-deficient ad libitum-fed (AL/TD) and dietary restricted (DR/TD) mice

The number of NeuN-immunoreactive neurons in AL/C mice was comparable to that of the DR/C mice (Figs. 1, 2). TD produced a significant reduction (60%) of the number of neurons in the submedial nucleus of AL/TD compared to ad libitum-fed controls (AL/C; P < 0.001).
The number of NeuN-immunoreactive neurons in the submedial nucleus of AL/C mice was 984±36, while that of AL/TD was 390±74 (Fig. 2).

TD also significantly reduced the number of NeuN-immunoreactive neurons in the submedial nucleus of DR/TD compared to dietary restricted controls (DR/C; P<0.01) (Figs. 1, 2). However, only a 31% reduction occurred in the DR/TD mice (687±35) compared to DR/C (990±19). Thus, DR attenuated the TD-induced neuronal loss in the submedial nucleus (P<0.01, DR/TD vs. AL/TD).

3.4. Heme oxygenase-1 (HO-1) induction in thiamine-deficient ad libitum-fed (AL/TD) and dietary restricted (DR/TD) mice

HO-1 (EC 1.14.99.3, also known as HSP32) is a marker for both nitrosative and oxidative stress [31]. Our previous studies demonstrate that HO-1 is induced in microglia in the thalamus during TD, and that the pattern of HO-1 induction overlaps with neuronal loss [8,10]. The severity of neuronal loss is directly proportional to HO-1 induction. To determine whether the attenuation of TD-induced neuronal loss by DR is associated with a reduction of oxidative damage, HO-1 induction was measured in the thalamus of DR/TD and AL/TD mice.

Light HO-1 immunoreactivity occurred throughout the...
brain in all mice (Fig. 3). No significant differences in the background level of HO-1 immunoreactivity were observed between the AL/C and DR/C groups. TD induced HO-1 in microglia within the thalamus of both AL/TD and DR/TD (Figs. 3, 4). However, DR attenuated the microglial HO-1 induction in the thalamus during TD. The number of HO-1-labeled microglia in the DR/TD group (27±2) was significantly lower than that of the AL/TD mice (75±6; P<0.001; Fig. 4). As in past studies [10], the specificity of the HO-1 staining was confirmed by the lack of staining when sections were incubated in preadsorbed antibodies (data not shown).

### 3.5. IgG extravasation in thiamine-deficient ad libitum-fed (AL/TD) and dietary restricted (DR/TD) mice

Consistent with our previous reports [7–10], IgG immunoreactivity did not accumulate in any brain region of either the AL/C or DR/C mice. However, varying degrees of the number of intensely stained microglia (arrows) in the thalamus of DR/TD compared to AL/TD. A high magnification of intensely stained cells, previously identified by colocalization studies as microglia (7) are shown (inset). Scale bar=250 μm, 20 μm for inset.

Fig. 4. HO-1 induction in AL/TD and DR/TD mice. Values represent the mean±S.E.M. of HO-1 labeled microglia in three rostrocaudal levels of the thalamus. *P<0.001 (vs. AL/TD).
of increases in IgG immunoreactivity occurred in the thalamus of both AL/TD and DR/TD mice. IgG accumulated more extensively in the thalamus in the four out of five AL/TD mice compared to DR/TD mice (Table 1). Overall, DR reduced the severity of IgG accumulation in the thalamus.

4. Discussion

A large body of evidence suggests that DR modulates free radical metabolism ([20] for review). The rationale behind testing the ability of DR to attenuate the TD-induced pathology hinges on the assumption that if oxidative stress plays a key role in selective neurodegeneration during TD, then DR should reduce neuronal loss in this model. Indeed, the current data demonstrate a neuroprotective effect of DR in TD. The attenuation of TD-induced loss of NeuN-immunoreactive neurons accompanied a dramatic reduction of HO-1 induction and IgG extravasation in the thalamus of DR/TD compared to AL/TD mice. Our previous work demonstrates that TD-induced increases in microglial HO-1, an indication of oxidative stress, correlate with the neuronal dropout [10]. In addition to reactive iron and ferritin elevation in microglia, the nitration product of peroxynitrite, nitrotyrosine [9], and the lipid peroxidation product, 4-hydroxynonenal [10] accumulate in neurons within the thalamus during late stages of TD. Interestingly, DR suppresses the age-related accumulation of the pro-oxidant iron [13], reduces kainate-induced 4-hydroxynonenal induction in hippocampal neurons in mice [36], and attenuates lipid peroxidation in mitochondrial and microsomal membranes [24].

DR may be neuroprotective against oxidative stress by inducing a low-level stress which enhances free radical scavengers that protect neurons from subsequent insults. DR upregulates several antioxidants including catalase, glutathione reductase, glutathione S-transferase, and superoxide dismutase [19,21]. During TD in ad libitum-fed animals, superoxide dismutase increases in microglia in vulnerable regions [33] as an antioxidant response. DR may enhance this normal neuroprotective mechanism. Thus, a DR-induced elevation of basal levels of superoxide dismutase may explain the attenuation of neuronal loss during TD.

The reduction of blood–brain barrier breakdown by DR in the TD thalamus may be due to the ability of DR to modulate vascular factors. We previously reported that TD induces intercellular adhesion molecule-1 (ICAM-1) and endothelial nitric oxide synthase (eNOS) in the vulnerable regions, and that gene-targeted disruption of ICAM-1 or eNOS attenuates neuronal dropout and HO-1 induction during TD [8]. These findings suggest that ICAM-1 and eNOS induction are critical events that lead to the TD-induced neurodegeneration. A recent human study documented that a 12-week caloric restriction reduces the levels of circulating endothelial adhesion molecules including ICAM-1 [15]. Thus, although the link between vascular factors and neurodegeneration is not well understood, modulation of endothelial oxidative stress may explain the neuroprotective effect of DR in the TD model.

Evidence for the beneficial effect of DR in a variety of models of neurodegeneration is accumulating. DR reduces neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson’s disease [14], excitotoxic and metabolic injury [4], and in mice with presenilin-1 mutation that is linked to early onset of Alzheimer’s disease [36]. N-methyl-D-aspartate-mediated excitotoxicity has been implicated in cell death in late stages of TD [23]. DR reduces stress proteins, such as the heat shock protein (HSP)-70 and glucose-regulated protein (GRP)-70, that can protect neurons against excitotoxic injury [1,2,14,26]. However, DR had no beneficial effect in transgenic mice expressing amyotrophic lateral sclerosis-linked Cu/Zn-superoxide dismutase mutations [30]. Thus, DR affords neuroprotection against the death-promoting action of oxidative stress in many, but not all, models of neurodegeneration. This suggests that the type of reactive oxygen species that is involved in different neurodegenerative disorders, and the type of reactive oxygen species that is suppressed by DR may differ.

The results from studies using animal models provide strong support for the beneficial effects of DR in human neurodegenerative disorders. Case studies reveal that a low caloric intake reduces the risk for Alzheimer’s disease [27] and Parkinson’s disease [25]. DR may be a significant approach for mitigating the neurodegenerative process that lead to diseases where oxidative stress plays a prominent role, such as Alzheimer’s and Parkinson’s diseases.

These current results suggest that oxidative damage is critical to neuronal vulnerability to TD, and that dietary restriction modulates the extent of free radical damage in

### Table 1

Dietary restriction and the region-specific increase in IgG immunoreactivity in the thalamus after TD

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal ID #</th>
<th>IgG extravasation score*</th>
</tr>
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<tbody>
<tr>
<td>AL/TD</td>
<td>1</td>
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<td>2</td>
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<td></td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>DR/TD</td>
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<td>+</td>
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<td></td>
<td>7</td>
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</tbody>
</table>

*Three sections 175 μm apart were scored based on the extent of IgG immunoreactivity.

A laterodorsal thalamic nucleus and dorsal lateral geniculate nucleus.

++ A laterodorsal thalamic nucleus, dorsal lateral geniculate nucleus and ventrolateral and ventral posteromedial thalamic nucleus.

+++ A laterodorsal thalamic nucleus, dorsal lateral geniculate nucleus, ventrolateral, ventral posteromedial and reticular thalamic nucleus.
the brain. Thus, TD is an important tool for testing aspects of the relationship between aging, oxidative stress and nutrition.

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