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Increased Cerebral Free Radical Production During Thiamine Deficiency

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Concentration of reactive oxygen species (ROS) and the antioxidant glutathione (GSH) was measured in thalamus and cortex after 13 and 14 days of pyriethamine-induced thiamine deficiency (PTD) in the rat. The concentration of ROS was significantly elevated in thalamus and cortex on day 14 when righting reflexes were absent and spontaneous seizures occurred. No significant changes in GSH concentration were observed in thalamus or cortex on either day of treatment. These findings suggest that increased formation of free radicals occurs during the more acute symptomatic stage of thiamine deficiency and may contribute to the structural damage described in this model of Wernicke's encephalopathy.

Keywords: Thiamine; free radicals; oxidative stress; encephalopathy

INTRODUCTION

Numerous theories have been proposed to explain the mechanisms responsible for the neuroanatomical damage produced by thiamine deficiency (Butterworth, 1993; Langlais, 1995; Witt, 1986). An excess production of free radicals is not one of these theories but this pathogenetic mechanism is suggested by the following observations. First, pathologic lesions in the pyriethamine-induced thiamine deficient (PTD) rat are associated with increased levels of glutamate and activation of the NMDA receptor (Hazell *et al.*, 1993; Langlais, 1995; Langlais and Mair, 1990; Langlais and Zhang, 1993). Activation of glutamate-NMDA receptors leads to free radical formation (Bondy and Lee, 1993) and NMDA agonists are particularly potent in stimulating the rate of generation of reactive oxygen species (ROS) in cerebral tissue (Bondy and Lee, 1993). Activation of the NMDA receptor has also been implicated in postischemic elevation of lipid peroxidation in hippocampus and transient ischemia elevates extracellular fluid (ECF) levels of both excitatory amino acids and rates of hydroxyl radical formation (Delbarre *et al.*, 1991). Second, reactive astrocytes and microglia, important sources of the free radical superoxide

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(Halliwell and Gutteridge, 1985), are frequently observed in vulnerable brain regions of PTD treated rats (Collins, 1967; Zhang *et al.*, 1995) and mice (Watanabe and Kanabe, 1978). Third, increased levels of lactate and reduced pH occur in areas susceptible to necrosis during acute episodes of thiamine deficiency (Hakim, 1984). These phenomena may contribute significantly to oxidative damage since decreased pH may aid in mobilizing 'free iron', a transition metal that is important in catalyzing free radical production. Finally, thiamine deficiency significantly impairs the activity of transketolase prior to the onset of behavioral symptoms and histological changes in brain (Giguere and Butterworth, 1987). Transketolase is a key enzyme of the hexose monophosphate (HMP) shunt responsible for the generation of NADPH. This nucleotide coenzyme is necessary for the maintenance of reduced glutathione (GSH), an important antioxidant and free radical scavenger. Previous studies have demonstrated significant reduction of GSH concentrations in erythrocyte and heart (Hsu and Chow, 1960) and in brainstem (McCandless and Schenker, 1968) of symptomatic thiamine deficient animals.

The present study examined free radical production and oxidative stress in the pyriethamine-induced thiamine deficient (PTD) rat model of Wernicke's encephalopathy (Langlais and Mair, 1990). The thalamus and frontoparietal cortex were examined since the former is highly susceptible to thiamine deficiency-induced necrosis while the latter appears relatively resistant to necrosis but does undergo edematous changes (Watanabe and Kanabe, 1978; Takahashi *et al.*, 1988) and white matter damage (Langlais and Zhang, 1995). Twenty six male Sprague-Dawley rats 8 weeks old (270-300 gm) were randomly assigned to one of the following treatments: PTD - each rat received daily injections of pyriethamine HBr (0.25 mg/kg, i.p. Sigma Chem. Co.) and fed thiamine deficient chow (Teklad Mills); CT - each control rat was fed a thiamine-deficient chow equal to the average amount consumed by PTD rats and given daily injections of thiamine HCl (0.4 mg/kg, i.p.). Separate groups of PTD treated rats were sacrificed on the thirteenth day of treatment (PTD-13, N=7) and fourteenth day (PTD-14, N=6) of treatment. A previous study of this PTD model has shown that within the thalamus the earliest evidence of morphological changes in the absence of cell loss is observed on the thirteenth day of treatment. On the fourteenth day of treatment and at the onset of seizures, excitotoxic and/or apoptotic degenerative changes and a small degree of neuronal loss are evident in a few discrete thalamic nuclei, i.e. the anteroventral and ventrobasal, while the other nuclei are relatively well-preserved (Zhang *et al.*, 1995). All animals in the PTD-13 group were displaying symptoms of weight loss, ataxia and 5/7 had impaired righting reflexes. In the PTD-14 group, all animals had marked difficulty with righting and 4/6 were sacrificed within 1 hr following the appearance of seizures. Groups of CT animals were sacrificed on day 13 (N=7) and day 14 (N=6) of treatment. Rats were lightly anesthetized by inhalation of CO₂, decapitated and the brains rapidly removed. A 2 mm coronal section of diencephalon was placed on a freezing plate (-20°C), the entire thalamus and overlying frontoparietal cortex were dissected and separately stored in microcentrifuge tubes at -70°C. Each tissue was weighed and homogenized in 10 vols. of 0.32 M sucrose and centrifuged (1800 x g for 10 min). The supernatant was then centrifuged at 31,500 x g for 10 min to yield a mitochondrial pellet (P2) and a supernatant (S2) fraction. The P2 pellet was subsequently resuspended in HEPES buffer to a concentration of 0.1 gequiv/ml. This method of

preparing subcellular organelles from frozen brain tissues has been shown to preserve structure and metabolic integrity (Dodd *et al.*, 1981). The final protein concentration of the P2 and S2 suspensions was 1.6-3.1 mg/ml.

The concentration of ROS was determined with a spectrophotometric assay in which 2',7'-dichlorofluorescein diacetate (DCFH-DA) is oxidized by reactive oxygen to the fluorescent 2',7'-dichlorofluorescein (DCF) (LeBel and Bondy, 1990). Fifty μ l of the P2 suspension was incubated with 5 μ M DCFH-DA in a final volume of 2 ml HEPES at 37°C for 15 min. Fluorescence was monitored (488 nm excitation/525 nm emission) before and after incubation. Autofluorescence (>11% of total) was corrected by the inclusion of blanks with no DCFH-DA. ROS was quantified from a DCF standard curve (0.05-1.0 mM) and results expressed as nmol DCF formed/h/mg protein. The concentration of the antioxidant, reduced glutathione (GSH), was determined in the S2 fraction by the fluorometric measurement of the reaction product of GSH and monochlorobimane (mBCl) as previously described (Shrieve *et al.*, 1988). Monochlorobimane (5 mM in ethanol) was added to 0.1 ml of the S2 suspension and 1.9 ml of HEPES buffer to a final concentration of 10 μ M. The suspension was incubated for 15 min at 37°C. The fluorescent product was measured at 395 nm (excitation) and 470 nm emission. Tissue GSH concentration was determined using a GSH standard curve and expressed as mM/mg protein.

Data from the controls were pooled and examined with the data from the PTD-13 and PTD-14 groups using repeated measures (thalamus, cortex) ANOVA. Analysis of ROS levels (Table) demonstrated a significant effect of treatment ($F[2,23]=8.745$, $p=.0015$) and area ($F[1,23]=14.371$, $p=.0009$) but no significant treatment X area interaction ($F[2,23]=1.090$, $p=.353$). As shown in the Figure, ROS levels were higher in thalamus and cortex of the PTD animals after 13 and 14 days of treatment. Post-hoc analyses (Tukey's), however, demonstrated that the level of ROS in thalamus and cortex of the PTD-14 group was significantly elevated compared to controls. The elevation of ROS within thalamus of the PTD-14 animals (135%) is quite toxic and lethal. At this stage of PTD in the rat, morphological changes suggestive of an excitotoxic or apoptotic degeneration are observed within the anteroventral, ventrolateral, ventroposterolateral and posterior nuclei of thalamus (Zhang *et al.*, 1995). Neuronal loss is minimal and approximately 15-20% of the neurons are affected. Midline nuclei, i.e., central medial, anteromedial, mediodorsal, paracentral and parafascicular are unaffected. Because the entire thalamus was dissected and examined as a whole, it is impossible to determine if these elevations reflect much higher levels of ROS in only the affected nuclei or more modest increases within larger regions of thalamus. The level of ROS within thalamus was also elevated in thiamine deficient animals examined 1 day earlier but these changes were smaller and not significantly different from controls. At this stage, excitotoxic-like morphological changes are limited to two thalamic nuclei, the gelatinosus and anteroventral (Zhang *et al.*, 1995). The progressive and significant increase in ROS within frontoparietal cortex is somewhat unexpected since this brain region does not undergo the severe neurodegeneration and necrotic changes observed in thalamus. However, swelling of astrocytes, splitting of myelin sheaths, swelling of the periaxonal space (Takahashi *et al.*, 1988), and degenerating white matter fibers (Langlais and Zhang, 1995) have been observed within rat cortex after 13 days of PTD treatment, at the

onset of impaired righting reflexes and prior to the more acute symptomatic stage characterized by seizures. Loss of neurons and shrinkage of frontoparietal cortex have also been reported in rats following recovery from symptomatic stages of thiamine deficiency (Krill and Homewood, 1993; Langlais and Savage, 1995).

Figure 1. Level (mean $\pm$ SEM) of reactive oxygen species (A) and reduced glutathione (B) in thalamus and frontoparietal cortex of pyrithiamine-induced thiamine deficient (PTD) rats after 13 days (PTD-13) and 14 days (PTD-14) of treatment. Values are expressed as percent of concentration determined in a group paired controls (CT). * $p < .05$, Tukey's post-hoc test, compared to Control.

Table 1. Concentration of Reactive Oxygen Species (ROS) and Reduced Glutathione (GSH)

Group	N	ROS (nmol DCF formed/hr/mg Prot.)		GSH (μ M/mg Protein)	
		Thalamus	Cortex	Thalamus	Cortex
Control	13	1.72 $\pm$ 0.24	2.04 $\pm$ 0.22	11.20 $\pm$ 1.74	9.57 $\pm$ 2.44
PTD-13	7	1.99 $\pm$ 0.29	2.33 $\pm$ 0.40	12.21 $\pm$ 0.95	10.44 $\pm$ 0.81
PTD-14	6	2.32 $\pm$ 0.38*	2.43 $\pm$ 0.28*	10.37 $\pm$ 1.93	9.77 $\pm$ 0.73

Values represent the mean $\pm$ S.D. of the concentration of ROS and GSH in thalamus and frontoparietal cortex of pyrithiamine-induced thiamine deficient rats after 13 days (PTD-13) and 14 days (PTD-14) of treatment and a group of paired controls. * $p < .05$ Tukey's post-hoc test, compared to Control.

Analyses of GSH levels (Table 1) demonstrated no significant treatment ($F[2,23]=1.752$, $p=.196$), nor interaction (treatment $\times$ area) effects ($F[2,23]=.528$, $p=.597$). There was a significant effect of area ($F[1,23]=7.854$, $p=.01$), reflecting the overall higher levels of GSH within thalamus compared to cortex. The absence of a significant decline in GSH levels suggest that increased free radical production may not occur in these regions. However, glutathione levels can undergo a biphasic response to oxidative stress and thus the absolute level of GSH may not be as reliable an index of oxidative stress as the direct measurement of ROS (Adams *et al.*, 1993).

The source of elevated free radical production during thiamine deficiency is unknown but at least two possibilities exist. First, a significant increase in ROS was observed only in the PTD-14 group in which most animals had developed spontaneous or sensory-evoked seizures. Seizures are known to elevate cerebral production of ROS (Armstead *et al.*, 1989; Bruce and Baudry, 1995; Ikeda and Long, 1990). However, seizures are often associated with excitotoxins which by themselves cause excessive production of oxygen free radicals through activation of NMDA receptors (Coyle and Puttfarcken, 1993). It has been shown that activation of NMDA receptors in neuronal cultures leads to the production of superoxide radicals (Lafon-Cazal *et al.*, 1993). Previous studies have demonstrated increased extracellular levels of the excitotoxin glutamate within thalamus prior to the onset of seizures in the PTD rat model (Hazell *et al.*, 1993; Langlais and Zhang, 1993). Since several of the thalamic nuclei affected by thiamine deficiency play critical roles in suppressing seizures (Gale, 1992), it is possible that damage to these regulatory neurons through increased glutamate NMDA activation and free radical production may precipitate the onset of seizures.

Other observations suggest that disturbances of vascular and glial cells may contribute to the increased levels of reactive oxygen species. Thiamine deficiency encephalopathy is often characterized as an early vascular-glial disorder which eventually culminates in neuronal loss and tissue necrosis. This hypothesis is supported by recent evidence of an early breakdown of the blood-brain barrier (BBB) and subsequent vasogenic edema in PTD rats (Calingasan *et al.*, 1995; Zhang *et al.*, 1995) and mice (Harata and Iwasaki, 1995). More importantly, breakdown of the BBB occurs selectively in vulnerable brain regions and precedes the onset of cytomorphological alterations of glia and neurons. The biochemical basis for thiamine deficiency and regional BBB breakdown is unclear. However, thiamine has been identified within the cytoplasm of endothelial cells, in glial processes surrounding the capillary wall, and in cell structures closely associated with the basement membrane (Gragera *et al.*, 1994). Endothelial cells are a primary source of nitric oxide (NO), a free radical which acts both as a molecular messenger and as a cytotoxin (Bredt and Snyder, 1994; Moncada *et al.*, 1991). NO alters the permeability of the BBB and causes structural damage to brain capillary endothelial cells (Au *et al.*, 1985) and surrounding tissue (Moncada *et al.*, 1991). NO is also produced by macrophages/microglia and astrocytes following activation of the inducible form of the synthetic enzyme nitric oxide synthase (iNOS).

The present results have important clinical and theoretical implications. First, they suggest that administration of antioxidants may be an effective treatment approach for the prevention or minimization of thiamine deficiency-induced damage to the brain. Second, they provide a biochemical basis for the synergistic interaction of thiamine deficiency and ethanol proposed in the etiology of alcohol related disorders. Increasing evidence suggest that free radicals, nitric oxide and NMDA receptors play key roles in the permanent structural and functional alterations observed after long term ethanol exposure (Lancaster, 1992; Pellmar, 1992). Finally, the present findings suggest that future studies are needed to examine the role of NO and other free radicals in the evolution of structural changes in more discrete regions thalamus, cortex and other vulnerable brain structures during thiamine deficiency.

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