Title
Increased cerebral free radical production during thiamine deficiency

Journal
Metabolic Brain Disease, 12(2)

ISSN
0885-7490

Authors
Langlais, Philip J
Anderson, Gary
Guo, SX
et al.

Publication Date
1997-06-01

DOI
10.1007/bf02674735

Metabolic Brain Disease, Vol. 12, No. 2, 1997

Increased Cerebral Free Radical Production During Thiamine Deficiency

Philip J. Langlais[1,2,4], Gary Anderson[2], S.X. Guo[3], Stephen C. Bondy[3]

Received: 27 June 1996; Accepted: 23 December 1996

Concentration of reactive oxygen species (ROS) and the antioxidant glutathione (GSH) was measured in thalamus and cortex after 13 and 14 days of pyrithiamine-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 occured. 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 pyrithiamine-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

[1] Dept. of Psychology, San Diego State University, San Diego, CA.

[2] Neurology Research, V. A. Medical Center, San Diego, CA

[3] Dept. of Community and Environmental Med., Univ. of California, Irvine, CA

[4] To whom correspondence should be addressed at Behavioral Neurobiology, San Diego State University, 6363 Alvarado Ct., Suite 237, San Diego, CA 92120, Tel: (619) 594-1816; FAX: (619) 594-8707; email: planglai@sunstroke.sdsu.edu

0885-7490/97/0600-0137$12.50/0

(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 pyrithiamine-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; Takahaski *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 pyrithiamine 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 thirtheenth 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 CO_2, 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 spectrophometric assay in which 2',7'-dichlorofluoroscein diacetate (DCFH-DA) is oxidized by reactive oxygen to the fluorescent 2',7'-dichlorofluoroscein (DCF) (LeBel and Bondy, 1990). Fifty µl of the P2 suspension was incubated with 5 µM 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 µM. 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 (Kril and Homewood, 1993; Langlais and Savage, 1995).

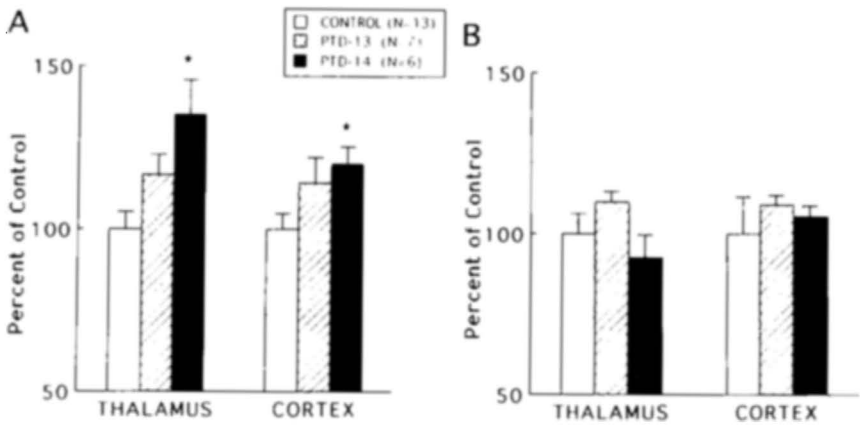

Figure 1. Level (mean ± SEM) of reactive oxygen species (A) and reduced glutathione (B) in thalamus and frontoparietal cortex of pyrithiamine-induced thiamine deficient (PTD) rats after 13 days (PTD-13) and 14 days (PTD-14) of treatment. Values are expressed as percent of concentration determined in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

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

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

Values represent the mean ± 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 pairfed 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 X 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 atleast 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.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of Health (AA 8281) and Dept. of Veterans Affairs.

REFERENCES

Adams, J.D., Wang, B., Klaidman, L.K., LeBel, C. P., Odunze, I.N. and Shah, D. (1993). New aspects of brain oxidative stress induced by tert-butylhydroperoxide. *Free Rad. Biol. Med.* **15**:195-202.

Armstead, W.M., Mirro, R., Effler, C.S. and Busija, D.W. (1989). Cerebral superoxide anion generation during seizures in newborn pigs. *J. Cerebr. Blood Flow Metab.* **9**:175-179.

Au, A.M., Chan ,P.H., and Fishman, R.A. (1985). Stimulation of phospholipase A2 activity by oxygen-derived free radicals in isolated brain capillaries. *J. Cell. Biochem.* **27**:449-453.

Bondy, S.C. and Lee, D.K. (1993). Oxidative stress induced by glutamate receptor agonists. *Brain Res.* **610**:229-233.

Bredt, D. and Snyder, S.A. (1994). Nitric oxide: a physiologic messenger molecule. *Ann. Rev. Biochem.* **63**:175-195.

Bruce, A.J. and Baudry, M. (1995). Oxygen free radicals in rat limbic structures after kainate-induced seizures. *Free Radical Biol. Med.* **18**:993-1002.

Butterworth, R.F. (1993). Pathophysiological mechanisms responsible for the reversible (thiamine-responsive) and irreversible (thiamine non-responsive) neurological symptoms of Wernicke's encephalopathy. *Drug and Alcohol Review* **12**:315-322.

Calingasan, N.Y., Baker, H., Sheu, K.-F.R., and Gibson, G.E. (1995). Blood-brain barrier abnormalities in vulnerable brain regions during thiamine deficiency. *Exp. Neurol.* **134**:64-72.

Collins, G. H. (1967). Glial cell changes in the brain stem of thiamine deficient rats. *Am. J. Pathol.* **50**:791-814.

Coyle, J.T. and Puttfarcken, P. (1993). Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262**:689-695.

Delbarre, G., Delbarre, B., Calinon, F., and Ferger, A. (1991). Accumulation of amino acids and hydroxyl free radicals in brain and retina of gerbil after transient ischemia. *J. Ocular Pharmacol.* **7**:147-155.

Dodd, P.R., Oakley, J.A., Edwardson, A.E., Perry, J.A., and Delaunoy, J.P. (1981). A rapid method for preparing synaptosomes: comparison with alternative procedures. *Brain Res.* **226**:107-118.

Gale, K. (1992). Subcortical structures involved in convulsive seizure generation. *J. Clin. Neurophysiol.* **9**:264-277.

Giguere, J.F. and Butterworth, R.F. (1987). Activities of thiamine-dependent enzymes in two experimental models of thiamine deficiency encephalopathy. *Neurochem. Res.* **12**:305-310.

Gragera, R.R., Muniz, E. and Martinez-Rodgriguez, R. (1994). Neuromediators in the cerebellar blood-brain barrier and its microenvironment. Immunocytochemical demonstration of taurine, glycine, serotonin, thiamin and AATase. *J. fur Hirnforschung* **35**:31-38.

Hakim, A.M. (1984). The induction and reversibility of cerebral acidosis in thiamine deficiency. *Ann Neurol.* **16**:673-679.

Halliwell, B., and Gutteridge, J.M.C. (1985). Oxygen radicals and the nervous system. *TINS* **8**:22-26.

Harata, N. and Iwasaki, Y. (1995). Evidence for early blood-brain barrier breakdown in experimental thiamine deficiency in the mouse. *Metab. Brain Dis.* **10**:159-174.

Hazell, A., Butterworth, R., and Hakim, A. (1993). Cerebral vulnerability is associated with selective increase in extracellular glutamate concentration in experimental thiamine deficiency. *J Neurochem.* **61**:1155-1158.

Hsu, G.M. and Chow, B.F. (1960). Effect of thiamine deficiency on glutathione contents of erythrocytes and tissues in the rat. *Proc. Soc. Exptl. Biol. Med.* **104**:178-185.

Ikeda, Y. and Long, D.L. (1990). The molecular basis of brain injury and brain edema: the role of oxygen free radicals. *Neurosurgery* **27**:1-11.

Kril, J.J. and Homewood, J. (1993). Neuronal changes in the cerebral cortex of the rat following alcohol treatment and thiamine deficiency. *J. Neuropath. Exp. Neurol.* **52**:586-593.

Lafon-Cazal, M., Pietri, S., Culcasi, M., and Bockaert, J. (1993). NMDA-dependent superoxide production and neurotoxicity. *Nature* **364**:535-537.

Lancaster, F. E. (1992) Nitric oxide and ethanol-induced brain damage--a hypothesis. In (W.A. Hunt and S.J. Nixon, eds.), *Alcohol-Induced Brain Damage*, National Institute of Health Publications, Washington, pp. 373-386.

Langlais, P.J. (1995). Pathogenesis of diencephalic lesions in an experimental model of Wernicke's encephalopathy. *Metab.Brain Dis.* **10**:31-44.

Langlais, P.J., and Mair, R.G. (1990). Protective effects of the glutamate antagonist MK-801 on pyrithiamine-induced lesions and amino acid changes in rat brain. *J Neurosci.* **10**:1664-1674.

Langlais, P.J. and Savage, L.M. (1995). Thiamine deficiency in rats produces cognitive and memory deficits on spatial tasks that correlate with tissue loss in diencephalon, cortex, and white matter. *Behav.Brain Res.* **68**: 75-89.

Langlais, P.J., and Zhang, S.H. (1993). Extracellular glutamate is increased in thalamus during thiamine deficiency induced lesions and is blocked by MK-801. *J. Neurochem.*. **61**:2175-2182.
Langlais, P.J., and Zhang, S.H. (1997). Cortical and subcortical white matter damage without Wernicke's Encephalopathy after recovery from thiamine deficiency in the rat. Alcohol Clin. Exp. Res. (in press)
LeBel, C.P. and Bondy, S.C. (1990). Sensitive and rapid quantitation of oxygen reactive species in rat synaptosomes. *Neurochem. Int.* **17**:435-440.
McCandless, D.W. and Schenker, S. (1968). Encephalopathy of thiamine deficiency: Studies of intracerebral mechanisms. *J. Clin. Invest.* **47**:2268-2280.
Moncada, S., Palmer, R.M.J., and Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *J. Pharmacol Exptl Ther.* **43**:109-142.
Pellmar, T.C. (1992). Do free radicals contribute to ethanol-induced synaptic damage? In (W.A. Hunt and S.J. Nixon, eds.), *Alcohol-Induced Brain Damage*, National Institute of Health Publications, Washington, pp. 339-354.
Shrieve, D.C., Bump, E.A. and Rice G.C. (1988). Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. *J. Biochem.* **263**:14107-14.
Takahashi, H., Nakazawa, S., Yoshino, Y. Shimura, T. (1988). Metabolic studies of the edematous cerebral cortex of the pyrithiamine-treated rat. *Brain Res.* **441**:202-208.
Watanabe, I., and Kanabe, S. (1978). Early edematous lesion of pyrithiamine induced acute thiamine deficient encephalopathy in the mouse. *J. Neuropath. Exp. Neurol.* **37**:401-413.
Witt, E.D. (1985). Neuroanatomical consequences of thiamine deficiency: a comparative analysis. *Alcohol & Alcoholism* **20**:201-221.
Zhang, S.X., Weilersbacher GS, Henderson SW, Corso T, Olney JW, and Langlais PJ. (1995). Excitotoxic cytopathology, progression, and reversibility of thiamine deficiency-induced diencephalic lesions. *J. Neuropathol. Exp. Neurol.* **54**:255-267.