Short Communication

Energy And Glucose Pathways In Thiamine Deficient Primary Rat Brain Microvascular Endothelial Cells

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Abstract. Thiamine deficiency (TD) results in lactate acidosis, which is associated with neurodegeneration. The aim of this study was to investigate this alteration in primary rat brain endothelia. Spectrophotometric analysis of culture media revealed that only a higher concentration of pyrithiamine, which accelerates the intracellular blocking of thiamine, significantly elevated the lactate level and lactate dehydrogenase activity within 7 days. The medium without pyrithiamine and with a thiamine concentration comparable to pathophysiological plasma levels mildly reduced only the activity of transketolase. This suggests that significant metabolic changes may not occur at the early phase of TD in cerebral capillary cells, while anaerobic glycolysis in capillaries may be mediated during late stage/chronic TD.

Key words: Endothelial cells — Energy metabolism — Lactate — Thiamine deficiency encephalopathy — Transketolase

Thiamine deficiency (TD) is associated with selective neuronal degeneration manifested in alcoholic patients suffering from Wernicke–Korsakoff syndrome. However, several aging disorders, such as Alzheimer's and Parkinson's disease, were also reported to exhibit these features (Calingasan et al. 1998). Thiamine is phosphorylated to thiamine diphosphate, a co-enzyme, upon which the three enzyme systems have been shown to be dependent: α-ketoglutarate dehydrogenase and pyruvate dehydrogenase involved in the citric acid cycle, and transketolase (TK) present in the pentose phosphate pathway. Therefore, a reduced level of thiamine in the brain accounts for low glucose oxidation (Butterworth 1989). Several authors have reported that the blood-brain barrier (BBB) may also be involved in the pathogenesis of TD encephalopathy (Watanabe et al. 1981; Calingasan et al. 1995; Harata and Iwasaki 1995; Calingasan and Gibson 2000) mainly due to the damage mediated through oxidative stress induction. The BBB is established by a single layer of endothelial

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cells joined together by fasciae occludentes lining the continuous capillaries (Gartner and Hiatt 1997). The recent study of Lockman et al. (2003) suggests, however, that significant thiamine uptake is unlikely related to brain capillary cells and that this mechanism is carried out *via* a carrier-mediated transport with a significant brain efflux. Nevertheless, BBB endothelia could non-specifically associate with thiamine *via* restricted permeation and the chronic lack of thiamine in patients could thus still affect energy/glucose pathways in BBB cells, which require supply of this vitamin. Because accumulation of lactate is considered as the most striking alteration in TD (Thauvin-Robinet et al. 2004) that causes neurodegeneration (Navarro et al. 2005), the current study explores these factors and TK activity in primary endothelial cells.

The rat brain endothelial cells were obtained as described previously (Hughes and Lantos 1986; Abbott et al. 1992) from gray matter of Sprague-Dawley rats, two months old (Charles River Breeding Farms, QC, Canada) after applying anesthesia with sodium pentobarbital. Treatment of animals conformed to the institutional guidelines regarding the care and use of animals for experimentation. The main separation step included isolation of digested capillary fragments by centrifugation on a Percoll gradient (Pharmacia Biotech). Fragments were then plated out on the 35 mm culture dishes, which were treated prior to the experiments with rat collagen type I (Sigma). Composition of the culture medium (Nobles et al. 1995) was modified as follows: Dulbecco's modified Eagle medium (DMEM; Invitrogen Life Technologies, Burlington, ON, Canada) was supplemented with 100 $U \cdot ml^{-1}$ penicilin, 100 $\mu g \cdot ml^{-1}$ streptomycin, 20% horse serum, 75 $\mu g \cdot ml^{-1}$ endothelial cell growth supplement (Sigma), 100 $\mu g \cdot ml^{-1}$ heparin grade I and 4% supplement containing vitamin C, glutathione, insulin, transferrin, and selenium. In order to induce TD, the custom-designed thiamine-free DMEM was supplemented as the above control medium and referred to as the thiamine-decreased medium, because 20% horse serum added to the thiamine-free medium resulted in the final concentration of 7.8 $nmol \cdot l^{-1}$ thiamine (Wilkinson 1993). This calculation was based on the fact that serum contains primarily the free form of thiamine. Pyrithiamine (Sigma), a thiamine antagonist, was added to the thiamine-decreased medium at the final concentration of 50 $\mu g \cdot ml^{-1}$ (119 $\mu mol \cdot l^{-1}$) to further accelerate reduction of intracellular thiamine levels. Control cells were cultured in regular DMEM supplemented as described above with the supplement containing thiamine hydrochloride (11.7 $\mu mol \cdot l^{-1}$ thiamine). Cells were cultured in 5% CO_2 at 37℃ and the presence of endothelial cells was confirmed by staining for von Willebrand factor (antibodies from DAKO Diagnostics, Mississauga, ON, Canada) by immunocytochemistry. After confluence reached 80%, the culture medium was replaced with the thiamine-decreased medium with or without pyrithiamine. At Day 3 and Day 7 of the treatment, media were removed and cells collected. Viable cell count was performed using 0.1% Trypan blue dye according to Adams (1990).

Lactate release and lactate dehydrogenase (LDH) activity assay were performed using the kits from Sigma for the blood samples and were adjusted for the medium samples. Absorbance of the samples was measured spectrophotometri-

cally at 340 nm. TK activity was assayed according to Dreyfus, who introduced the method based on the determination of color reaction of sedoheptulose 7-P with the cysteine-sulfuric acid. Total heptose values were evaluated as the difference in absorbance between 510 and 540 nm and expressed as μmol or nmol of sedoheptulose *per* mg of protein$\cdot$min^{-1}. The protein content in cell lysates was measured using colorimetric spectrophotometry (Bio-Rad Laboratories, Mississauga, ON, Canada) read at 600 nm according to the manufacturer. The results from three experiments were calculated by a two-way analysis of variance (ANOVA) followed by the Fisher LSD test of Statistica Program (StatSoft) to assess the difference within the groups. Results are presented as means $\pm$ S.E.M.

Staining for the endothelial specific marker, von Willebrand factor, confirmed that contamination of the monolayer varied from 2% to 9% in several isolation procedures. Contaminating cell types were recognized according to the original protocol (Abbott et al. 1992). If contaminating cells affected the experimental results, the statistical analysis should resolve possible variation.

In order to examine lactate amount released, the media from treated cells were measured at Day 3 and Day 7. Within both incubation periods, pyrithiamine caused a significant increase in lactate release corresponding to 0.9 $\pm$ 0.026 and 1.2 $\pm$ 0.026 mmol$\cdot$l^{-1} when compared to that in the media from control cells showing 0.6 $\pm$ 0.026 and 0.7 $\pm$ 0.028 mmol$\cdot$l^{-1}, respectively ($p < 0.01$; Fig. 1A). Treatment of cells with the thiamine-decreased medium without pyrithiamine did not result in an increase in lactate levels during the treatment. These data corresponded to 0.572 $\pm$ 0.064 and 0.754 $\pm$ 0.055 mmol$\cdot$l^{-1} within Day 3 and Day 7, respectively. The differences obtained from the treatment with and without pyrithiamine were also significant ($p < 0.01$; Fig. 1A).

In order to monitor cell damage, the media were assayed for LDH activity. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay, which was shown to be sensitive to cellular stress induction not associated with cell death (Ham and Skoryna 2004), was not employed. Therefore, Trypan blue count was utilized to confirm cell viability. Pyrithiamine caused a significant increase in LDH activity at Day 3 (89 $\pm$ 6.82 U$\cdot$ml^{-1}) and also at Day 7 (121 $\pm$ 1.66 U$\cdot$ml^{-1}) when compared to the controls (57 $\pm$ 1.11 and 58 $\pm$ 3.82 U$\cdot$ml^{-1}; $p < 0.01$; Fig. 1B). This corresponds to 95 $\pm$ 0.86 and 89 $\pm$ 1.44% viability in pyrithiamine-treated cells at Day 3 and Day 7, respectively ($p < 0.05$; Fig. 1C). Cells cultured in the thiamine-decreased medium without pyrithiamine showed neither an increase in the LDH activity (62 $\pm$ 8.40 and 61 $\pm$ 6.82 U$\cdot$ml^{-1}), or a reduction in cell viability (97 $\pm$ 0.38 and 97 $\pm$ 0.57%) within both periods (Fig. 1B), when compared to the control values. The variability in LDH activities between the thiamine-decreased medium with and without pyrithiamine was also significant within both periods ($p < 0.01$), and the corresponding difference in cell viability was significant only at Day 7 ($p < 0.05$).

A small but significant decrease in TK activity ($p < 0.01$) was monitored in the pyrithiamine-treated cells at Day 3 (77 $\pm$ 3.20% equal to 3.87 μmol$\cdot$mg^{-1} protein$\cdot$min^{-1}) and this reduction has been maintained by the end of incubation

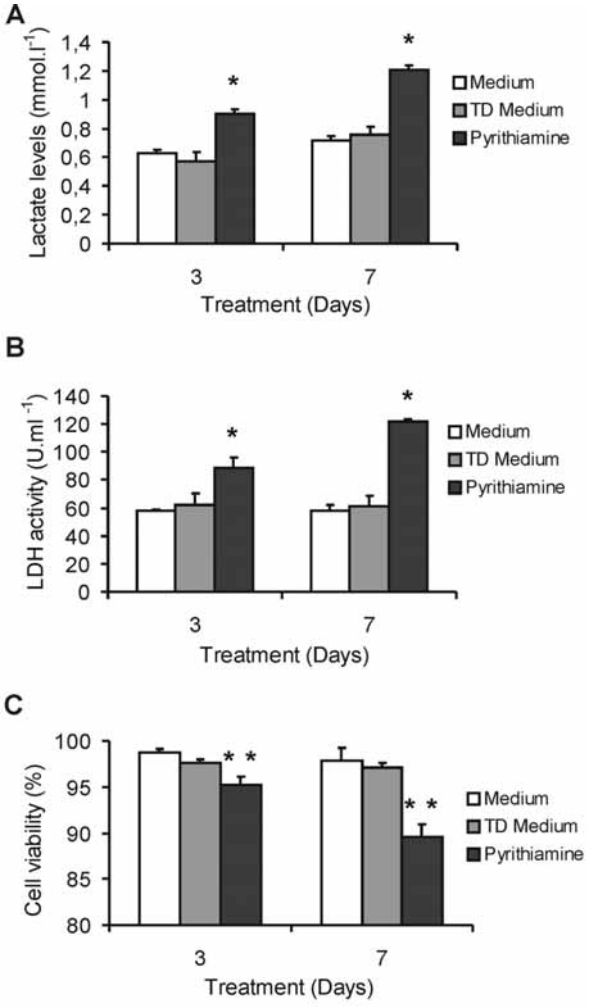

Figure 1. Effect of thiamine deficiency (TD) on lactate level (A), lactate dehydrogenase (LDH) activity (B), and cell viability in primary rat brain endothelial cultures (C). Cells were isolated according to Abbott et al. (1992) and subjected to the treatment with and without 119 μmol·l^{-1} pyrithiamine in the thiamine-free DMEM supplemented with 20% horse serum (TD Medium). Controls represented the cells cultured in normal DMEM containing the regular amount of thiamine 11.7 μmol·l^{-1} also supplemented with 20% serum (Medium). At Day 3 and Day 7, media were collected and lactate level (A) and LDH activity (B) were spectrophotometrically assayed using the kits from Sigma. Cell viability was estimated with 0.1% Trypan blue dye (C). Statistical analysis was computed with ANOVA followed by the Fisher LSD test for $n = 3$–4. Results are presented as means $\pm$ S.E.M. (* $p < 0.01$, ** $p < 0.05$ compared to controls). Differences in the lactate and LDH values between the thiamine-decreased conditions and pyrithiamine treatment were significant ($p < 0.01$) and related cell viability was significant only at the end of incubation ($p < 0.05$).

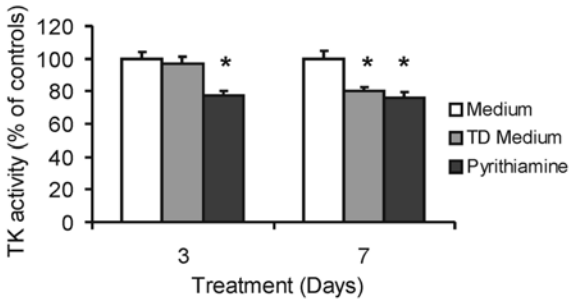

Figure 2. Effect of thiamine deficiency (TD) on transketolase (TK) activity in primary rat brain endothelial cultures. Primary cultures were prepared according to Abbott et al. (1992) and treated as indicated in Fig. 1. TK activity was estimated according to Dreyfus (1962) based on the activity of μmol or nmol sedoheptulose *per* mg protein$\cdot$min^{-1}. At Day 3, reduction in TK activity by pyrithiamine represented 77 $\pm$ 3.20% equal to 3.87 μmol *per* mg protein$\cdot$min^{-1} and at Day 7, a decrease in TK activity showed 75 $\pm$ 3.85% that is 3.94 μmol *per* mg protein$\cdot$min^{-1}. Controls were set to 100 $\pm$ 4.43% equal to 5.21 μmol *per* mg protein$\cdot$min^{-1}. The medium without pyrithiamine mediated a TK activity decrease 80 $\pm$ 2.18% corresponding to 4.18 μmol *per* mg protein$\cdot$min^{-1} only at Day 7. Results are presented as means $\pm$ S.E.M. (* $p < 0.01$ compared to controls for $n = 3$).

(75 $\pm$ 3.85% equal to 3.94 μmol$\cdot$mg^{-1}protein$\cdot$min^{-1}), when compared to the control groups (100 $\pm$ 4.43%, 5.21 μmol$\cdot$mg^{-1} protein$\cdot$min^{-1}; Fig. 2). The treatment of cells with the thiamine-decreased medium without pyrithiamine delayed the effect of TD on TK activity. Cells cultured in this medium reached a small decrease in TK activity only at the end of incubation and this reduction was approximately equal to that in the cells subjected to the pyrithiamine treatment. This represented 80 $\pm$ 2.18% of controls corresponding to 4.18 μmol$\cdot$mg^{-1} protein$\cdot$min^{-1} ($p < 0.01$; Fig. 2). The significant difference between the treatments with and without pyrithiamine was only monitored in the middle of the incubation ($p < 0.01$).

Thus, the current results imply that while *in vitro* short-term decrease in the thiamine level in culture medium could only mildly affect the energy pathways in brain capillary cells, an increased lack of thiamine through a thiamine antagonist may significantly affect anaerobic glycolysis in these cells. Thiamine predominates intracellularly in the form of thiamine diphosphate and the free form occurs mainly in plasma that contains 10% of the total thiamine. Thiamine plasma levels in healthy subjects are in the range from 0.018 to 0.855 μmol$\cdot$l^{-1} (Lee et al. 2000; Talwar et al. 2000; Mancinelli et al. 2003) and when compared to the normal DMEM, this medium represents a 13-fold increase over the upper level of presented physiological concentrations. Our thiamine-decreased conditions thus represented approximately a 2–100-fold decrease when compared to physiological thiamine status and a 9–16-fold decrease when compared to chronic alcoholic patients at risk of TD with plasma thiamine concentrations of 0.069–0.127 μmol$\cdot$l^{-1} (Lee et al. 2000; Mancinelli et al. 2003). These conditions affected only the cellular TK activ-

ity without influencing both the lactate level and LDH activity. The reduction in TK activity was not substantially changed even by pyrithiamine.

Administration of pyrithiamine into rats exerts the neurological signs of Wernicke's encephalopathy and, therefore, we also treated cells with this thiamine antagonist. Pyrithiamine accelerates intracellular reduction of thiamine (Giguere and Butterworth 1987) within a culture period, the condition that mainly occurs in an organism chronically suffering from TD. Because the low pyrithiamine concentrations did not significantly influence metabolic pathways in cultured capillary cells, we used a higher concentration of pyrithiamine. This level affected lactate release probably due to a decline in the ability of the cells to use this metabolite because of diminished citric acid cycle activity. The two enzymes present in this cycle, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, are thiamine-dependent (Butterworth 1989). Modest cell death was also associated with this pyrithiamine concentration, which further shows that BBB cells are not as vulnerable as neurons are to TD (Navarro et al. 2005). This cell death could be associated not only with a decreased pH level due to lactate, but also with other factors, which are induced by the oxidative stress (Calingasan and Gibson 2000). The level of lactate release from primary cells is in agreement with that from the immortalized rat brain microvessel endothelial RBE4 cell line and which was linked to the possible disruption of BBB integrity (Romero et al. 1997). However, the RBE4 cells showed a substantially higher (3-fold) lactate release than did the primary cells in our study (1.6-fold increase). Certain cell lines produce a greater acidic environment in comparison with normal cells, maybe because the glycolytic pathway in the cell lines produces pyruvate more rapidly than the citric acid cycle can accommodate (Voet and Voet 1995).

In conclusion, the present study implies that, in culture, only severe TD could cause significant anaerobic glycolysis in primary capillary cells, and this metabolic alteration may occur in the cerebral capillaries chronically deficient from thiamine. To elucidate whether or not these metabolic changes occur under such conditions, additional *in vivo* studies would be appropriate.

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