Low Energy Levels in Thiamine-Deficient Encephalopathy

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Abstract. Pyrithiamine-induced acute thiamine-deficient encephalopathy was produced in adult male Wistar rats. Twenty-four hours before the onset of neurological signs the brain showed no morphological abnormalities. Encephalopathic rats had symmetrical lesions of edematous necrosis localized in the thalamus, mammillary body, and pontine tegmentum. Biochemically, encephalopathic rats had brain thiamine levels less than 20% of controls. For the assay of the concentrations of adenosine triphosphate (ATP) and phosphocreatine, the brains were fixed using 5 KW microwave irradiation and were divided into four parts: cerebral cortex, diencephalon, lower brainstem, and cerebellum. In the lower brainstem of the encephalopathic rats ATP concentrations were 89.5% of normal controls. Phosphocreatine levels were lowered to 70% of controls in the diencephalon and to 75% in the lower brainstem. Total high energy phosphate levels were decreased to 89% of controls in the diencephalon and 91% in the lower brainstem before the onset of neurological signs and to 76% and 79%, respectively, after the onset. In the cerebral cortex and cerebellum high energy phosphates were not significantly reduced. Lower high energy phosphate levels and the distribution of edematous lesions were coincident in the brain. These findings suggest that a low energy state is closely related to the formation of edematous lesions in thiamine-deficient encephalopathy.

Key Words: Adenosine triphosphate; Brain edema; Phosphocreatine; Thiamine deficiency; Wernicke's encephalopathy.

INTRODUCTION

An animal model of the Wernicke-Korsakoff syndrome (thiamine-deficient encephalopathy in humans) can be produced quickly in rodents by the combined administration of a thiamine-deficient diet and pyrithiamine, an antagonist of thiamine (1). Initial changes in the brain include the edematous swelling of astrocytes, oligodendrocytes, myelin sheaths and neuronal dendrites (2). When the edema involves a large number of these cells, focal spongy lesions develop, where neuronal perikarya are also involved (3). Petechial hemorrhages can sometimes be seen due to blood vessel damage (4). Such lesions occur symmetrically in the thalamus, mammillary body and pontine tegmentum, but rarely in other areas.

The mechanism of these changes in thiamine-deficient encephalopathy is as yet unknown. It has been postulated that the intracellular edema is a result of decreased activity of Na-K-ATPase secondary to reduced ATP production in the glial cells and

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neurons (3, 5). This hypothesis remains unsubstantiated, because other investigators have reported that the brain ATP levels were not reduced (6–9).

After 5 KW microwave irradiation for rapid fixation of the brain significant reductions of ATP and phosphocreatine levels were seen in brains with this encephalopathy (10). This paper deals with detailed biochemical and morphological studies on the low energy state in thiamine-deficient encephalopathy.

**Materials and Methods**

Pyrithiamine-Induced Acute Thiamine-Deficient Encephalopathy in the Rat (PIATDER)

Nine-week-old, male Wistar rats weighing about 200 g each (SANKYO LAB. SERVICE, Tokyo, Japan) were fed *ad libitum* with a thiamine-deficient diet (Thiamine Test Diet, Low; TEKLAD TEST DIET, Madison, WI, U.S.A.) and were administered 0.1% pyrithiamine (SIGMA, St. Louis, MO, U.S.A.) daily i.p. at a dose of 1 mg/kg of body weight. All the rats developed encephalopathy on day 11 or 12, and were killed about 24 hours (h) after the onset of neurological signs (encephalopathic group). To obtain brains immediately before the onset of clinical signs (preclinical group) the rats were killed on day nine. The following three groups were used as controls: 1) thiamine-containing, normal diet group, 2) normal diet plus injection group, and 3) thiamine-deficient diet group.

**Morphological Study**

Ten preclinical, ten encephalopathic and five control rats on a thiamine-containing diet were examined. The rats were anesthetized with ether, then their brains were fixed by intracardiac perfusion with a cold solution containing 2% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). For electron microscopy, small tissue blocks were postfixed with 1% osmium tetroxide in the same buffer, dehydrated in a series of graded concentrations of ethanol, passed through propylene oxide and embedded in Epon 812. Semithin sections were stained with 1% toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Hitachi H-700 electron microscope.

**Biochemical Studies**

1. *Thiamine Levels in the Blood and Brain*: To determine the blood thiamine levels, 54 rats were divided into PIATDER and three control groups, as described above. On every second or fourth day, venous blood was collected from three rats from each of the four groups. Since the phlebotomy often caused anemia by blood loss, only non-anemic rats were chosen for bleeding. Each blood sample was kept frozen at −80°C until used. Thiamine levels in whole blood were determined by a thiochrome fluorescence method (11).

To study the brain thiamine concentrations, 18 rats were divided into three groups of six rats: encephalopathic group, thiamine-deficient diet control group and thiamine-containing diet control group. The body weight of each animal was monitored daily until it was killed. Immediately after decapitation, the brain was obtained by craniotomy and dissected on ice into three parts: 1) cerebral cortex, 2) cerebellum, and 3) brainstem including the basal ganglia, diencephalon, mesencephalon, pons and medulla oblongata. From each part of the brain, a 5% w/v homogenate was made in distilled water and was kept at −80°C. Thiamine concentrations were determined by the same method as for the blood thiamine.

2. *Energy Levels of the Brain*: The brains of ten preclinical and ten encephalopathic rats were fixed by focused microwave irradiation of the head for 1.5 seconds in an oven delivering 5 KW at 2,450 MHz (Model NJI 2601, NEW JAPAN RADIO CO., Tokyo, Japan). Twenty control rats of the thiamine-containing diet group were also treated in the same way. The brain was immediately removed and dissected on ice into four parts: 1) cerebral cortex, 2) diencephalon including the thalamus, mammillary body, upper mesencephalon and caudal striatum, 3) lower brainstem composed of lower mesencephalon, pons and medulla oblongata, and 4) cerebellum. The materials were frozen on dry-ice, weighed and homogenized in 6%
perchloric acid at 0°C with a Polytron homogenizer (KINEMATICA, GmbH, Switzerland). After centrifugation of the homogenates at 6,000 rpm for ten minutes (min) at 0°C, the supernatant was neutralized to pH 7.1–7.4 with 2.5 M potassium carbonate. The samples were then recentrifuged at 6,000 rpm for ten min at 0°C. The supernatant was collected and diluted 10-fold with distilled water. The concentrations of ATP and phosphocreatine were determined enzymatically following the methods of Lamprecht et al (12, 13) using a Hitachi H-650-60 fluorescence spectrophotometer. High energy phosphate levels were calculated by summing the values for the ATP and phosphocreatine. Since seizures are known to cause a reduction in energy levels of the brain, rats which convulsed during the microwave irradiation were excluded from this study. After the microwave irradiation, most brains were evenly and sufficiently fixed as judged by gross examination. In some unevenly fixed brains, only sufficiently fixed parts were collected and used for this study.

RESULTS

Behavioral Changes

The pyrithiamine-treated rats started to show anorexia, loss of weight and emaciation from day five (Fig. 1). On days 11 and 12 they suddenly developed characteristic neurological signs consisting of ataxia, stimulus-sensitive tonic seizures, opisthotonus and loss of righting reflexes. These signs fully developed within 24 and 48 h after onset. None of the control animals developed these abnormalities. While the thiamine-deficient control rats showed slight loss in body weight, the pyrithiamine-injected thiamine-fed controls did not.

Gross Findings

The brain and viscera were grossly normal except for rare petechial hemorrhages in the brain.

Fig. 2. Spongy lesion in the vestibular nucleus of pons (arrows); IV: fourth ventricle. Paraffin section, hematoxylin and eosin. × 40.

Fig. 3. Edematous astrocytes (a) in the mammillary body. Nerve cells (n) are intact. Semithin Epon section. Toluidine blue. × 280.

Light Microscopic Findings

No abnormalities were noted in the brains of the control and preclinical groups. In the encephalopathic rats, spongy changes were seen symmetrically in the thalamus, in the mammillary body, and lateral vestibular nucleus of the pontine tegmentum (Fig. 2). In the milder lesions where spongiosis was slight only astrocytes and oligodendrocytes were edematosus swollen; nerve cells and the blood vessels were preserved (Fig. 3). Severely edematosus lesions were rare and were characterized by extensive intracellular edema involving astrocytes, myelin sheaths, dendrites and nerve cell soma. Petechial hemorrhages were rarely seen even in these lesions.

Electron Microscopic Findings

In the milder lesions, edematous changes were seen only in astrocytes and oligodendrocytes. Some of them were necrotic with a loss of nuclear substance and cytoplasmic organelles. In the more advanced lesions myelin sheaths were also edematous in the periaxonal-submyelinic area. Edematous swelling was also noted in many dendrites and some neuronal perikarya. No abnormalities were observed in the blood vessels (Fig. 4).

Rats in the preclinical group showed no abnormalities in the thalamus, mammillary body or pontine tegmentum.

Biochemical Findings

1. Thiamine Levels: The blood thiamine concentration of the PIATDER group decreased rapidly until the onset of neurological signs when it fell to a level less than one-fifth of that measured in the thiamine-containing diet control rats (Fig. 5). The
Fig. 4. Edematous astrocytes in the mammillary body. $\times 2,600$.

Fig. 5. Blood thiamine levels. $\bullet--\bullet$, P.I.A.V. group; $\bullet--\bullet$, thiamine-deficient diet control group; $\Delta--\Delta$, thiamine-containing diet plus pyrithiamine injection group; $\Delta--\Delta$, thiamine containing diet group. Each point represents the mean $\pm SE$ (n-g/g) of three rats.
control rats on the thiamine-deficient diet experienced a similar decrease in their blood thiamine levels. The pyrithiamine-injected thiamine-fed controls showed an insignificant decrease in blood thiamine.

The brains of the control rats on the thiamine-containing diet had thiamine values in their three dissection areas averaging 3.95 μg/g wet weight. The value in the cerebellum was slightly higher than in the other parts of the brains as previously reported (14), but it was slightly reduced in the thiamine-deficient diet controls. In the encephalopathic group, the thiamine concentrations were as low as 8 to 17% of those in the thiamine-containing diet controls (Fig. 6). The extent of thiamine loss in the PIATDER group was, however, not significantly different in the cerebral cortex, brainstem or cerebellum.

2. Energy Levels: The brain ATP levels in the preclinical group did not significantly differ from the control rats. The encephalopathic group had decreases of 10.5% in the brainstem while the values of the other parts of the brain were unchanged (Fig. 7).

Phosphocreatine levels were not significantly altered in the preclinical group. In the encephalopathic group, however, the phosphocreatine levels were reduced by 30.2% in the diencephalon and by 25.4% in the brainstem (Fig. 8).

Total high energy phosphate levels in the preclinical group were reduced by 11% in the diencephalon and 9% in the brainstem (Fig. 9). After the onset of neurological signs, these levels were reduced by 24% of the controls in the diencephalon and 21% in the brainstem (Fig. 10). These levels were not changed significantly in the cerebral cortex and cerebellum throughout the course of the disease.
Figs. 7-10. Energy levels of PIATDER. Each value represents the mean ± SE (μmole/g). A, cerebral cortex; B, diencephalon; C, brainstem; D, cerebellum; □, PIATDER; □, control.

Fig. 7. ATP levels of the encephalopathic group. PIATDER, n = 8; control, n = 10.

Fig. 8. Phosphocreatine levels of the encephalopathic group. PIATDER, n = 8; control, n = 5.
Fig. 9. High energy phosphate levels in the preclinical group. PIATDER, n = 10; control, n = 5.

Fig. 10. High energy phosphate levels in the encephalopathic group. PIATDER, n = 8; control, n = 5.
In this study, the blood thiamine levels did not correlate with the progression of clinical signs in PIATDER. Even in the asymptomatic control rats on a thiamine-deficient diet, the blood thiamine levels were as low as those of the PIATDER group. In contrast, the brain thiamine levels were significantly reduced in the latter but not in the former. Thus, the reduction of brain thiamine levels seemed related to the development of encephalopathy. Within the same brain, however, tissue thiamine did not correlate well because of similar reductions in both the unaffected cerebral cortex and the affected brainstem.

The basic pathological change in thiamine-deficient encephalopathy was suggested to be an increase of vascular permeability (15). Recent studies reveal, however, that vascular permeability is not significantly changed at the initial stage (3, 6, 17), when morphologic changes show simply edema of astrocytes, oligodendrocytes, myelin sheaths and neuronal dendrites. The vascular changes, then, may be attributed to brain cell damage rather than vascular dysfunction (18). Although many biochemical abnormalities in the thiamine-deficient encephalopathy have been reported (19–24), no correlation of these chemical changes with morphologic alterations has been established. Thus, the pathogenetic mechanism of intracytoplasmic edema in thiamine-deficient encephalopathy remains unknown.

This study found low energy levels in the brains of animals with thiamine-deficient encephalopathy, while other investigators have shown normal or slightly elevated ATP and phosphocreatine levels for this condition (6–9). This discrepancy could be explained by two methodological differences; one in the experimental design and the other in the brain fixation method. First, our pyrithiamine encephalopathy (PIATDER) progressed more rapidly and severely than the diet-induced encephalopathies used by others. Accordingly our measured biochemical changes, such as reduced high energy levels, would be more advanced and discrete. Second, brain fixation by microwave irradiation, which we used, yielded faster inactivation of intermediary metabolites than the immersion fixation in liquid nitrogen used by other investigators. Since ATPase and creatine phosphokinase were inactivated in a shorter time period (less than 1.5 seconds), loss of ATP and phosphocreatine was small and their measured levels were closer to the true values. In our preliminary study, we also used liquid nitrogen to freeze the brains of eight encephalopathic rats and ten controls. Although the ATP and phosphocreatine levels approximated those measured on the microwave-irradiated rat brains, the standard error of the means (SEM) were not adequate. Sufficiently small SEM were obtained when the brains were fixed with microwave irradiation. In addition, microwave-irradiated brains made possible more accurate dissection of the brain tissue, whereas cutting the brains frozen in liquid nitrogen was more difficult due to its consistency (26–31).

The brain lesions in this study consisted of edematous necrosis only, whereas hemorrhage, the most common finding of PIATDER in other reports (4, 32), was rarely observed. This made the assay more reliable, because hemorrhages per se would hamper the determination of the energy loss directly related to the low thiamine supply. There is no clear explanation for the low incidence of hemorrhage in our experiment since there were no critical experimental differences between this study and others. It is possible that the Wistar rats used in this study had a different responsiveness to pyrithiamine, this trait having been acquired while they were maintained over generations in isolated colonies in Japan. Mild neurological signs
corresponded well to the non-hemorrhagic lesions in these animals. In any case, these rats seemed much better suited for these biochemical studies.

It is unlikely that the concentration of tissue constituents, such as ATP, might be diluted by edema and therefore result in low values per wet weight, because the brain blocks contained only a small volume of edematous lesions compared to a large normal part, as determined morphologically. The fact that the decrease of energy levels also occurred in the preclinical, morphologically intact brains ruled out the effect of edema. In a previous study (25), the protein concentrations per wet weight measured in the tissue blocks containing edematous lesions were not changed significantly. Accordingly, the reported levels on a wet weight basis, not on per protein weight, are still reliable values. (In fact, we used a large volume of tissue for the measurement of high energy phosphates and sacrificed the protein measurements.)

Low concentrations of ATP and phosphocreatine in the brain might generally indicate either a decrease in ATP production or an increase in ATP utilization. In the PIATDER animals, significant decreases of these high energy phosphates would have resulted from a decrease of ATP production because, as had been reported, the thiamine-dependent enzymes (19–24) and the glycolysis (33, 34) were decreased in the thiamine-deficient encephalopathy. However, this assumption must be confirmed after ATP and phosphocreatine turnover rates are estimated.

The energy levels in the brain were also markedly reduced 24 h after the onset of neurological signs, when edematous necrosis was already present in the brain. Although the ATP and phosphocreatine levels before the onset were not significantly reduced by separate measurements, the sum of these two high energy phosphate levels was significantly reduced. This indicated the presence of a low energy state in the preclinical stage, when morphologic changes of edema had not yet begun.

The decrease in the energy levels was seen only in the tissue blocks containing morphological lesions of edematous necrosis, but not in the morphologically intact tissue, such as the cerebral cortex and cerebellum. Thus, the distribution of the edematous lesions coincided with that of the focal energy loss in the brain. Conceivably, in the individual lesions, actual reduction of energy levels was far more severe than the measured values. However, the separation of these minute lesions from a large volume of the surrounding normal tissue was extremely difficult.

Because of these findings, it was concluded that the reduction of high energy phosphates in the encephalopathic areas was the cause, rather than the result, of edematous lesions of pyridoxine-induced acute thiamine-deficient encephalopathy.

One might speculate, then, from this data on the pathogenetic mechanism of intracytoplasmic edema in thiamine-deficient encephalopathy. Pyridoxine, an antagonist of thiamine, is considered an inhibitor of phosphorylation of thiamine to thiamine pyrophosphate (TPP), that acts as a cofactor of pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase and transketolase (35). Pyruvate oxidation in glycolysis and other oxidations in the TCA cycle are the main sources of energy, ultimately in the form of ATP, for the cerebral function. A decrease in TPP induced by pyridoxine would bring about a low concentration of ATP and phosphocreatine resulting in the reduced activity of Na-K-ATPase, a regulator of active transport of water and electrolytes in cell membranes. Intracellular edema would therefore result from this decreased ATPase activity. This view is supported by the finding that ouabain, a direct inhibitor of Na-K-ATPase, caused a similar swelling of the astroglia in vivo and in vitro (36–38). It has been shown that the activity of Na-K-ATPase

was somewhat higher in astrocytes (39, 40) and dendrites (41, 42) than in neurons. This would explain, then, why the edematous swelling in PIATDER occurred more severely in astrocytes and dendrites than in neurons.

In PIATDER the symptomatic animals had a uniform decrease in the thiamine concentration in the cerebral cortex, brainstem and cerebellum. The ATP and phosphocreatine levels, however, did not follow a similar distribution pattern but instead showed a decrease in the diencephalon and brainstem, but not in the other regions. Furthermore, there was also a close correlation between the location of the histopathologic lesions and the decrease of the ATP and phosphocreatine concentrations, but the decrease of thiamine did not correlate with this location. These findings indicate that thiamine-dependent energy metabolism in these affected regions was highly sensitive to thiamine deficiency and resulted in such selective vulnerability.

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REFERENCES

24. Platt BS, Lu GD. Studies on metabolism of pyruvate acid in normal and vitamin B1-deficient states; accumulation of pyruvic acid and other carbonyl compounds in beriberi and effect of vitamin B1. Biochem J 1939;33:1525-37
28. Medina MA, Jones DJ, Stavinoha WB, Ross DH. The levels of labile intermediary metabolites in mouse brain following rapid tissue fixation with microwave irradiation. J Neurochem 1975;24:225-7

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