Université de Montréal

Augmentation sélective de l'expression protéique et de l'ARNm de la synthase du monoxyde d'azote dans les régions vulnérables du cerveau chez les rats déficients en thiamine

par

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Ce mémoire intitulé :

Augmentation sélective de l'expression protéique et de l'ARNm de la synthase du monoxyde d'azote dans les régions vulnérables du cerveau chez les rats déficients en thiamine.

présenté par :

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D[r] Gilles Pomier- Layrargues,	président-rapporteur
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D[r] Allan Hazell,	membre du jury

Résumé

La déficience en thiamine entraîne la mort sélective de cellules neuronales dans les structures thalamiques.

Des études antérieures mettent en évidence le rôle du monoxyde d'azote dans la pathogenèse de la mort cellulaire causée par cette déficience vitaminique. Dans le but d'éclaircir l'origine de l'augmentation du monoxyde d'azote (NO), nous avons mesuré l'expression des isoformes de l'oxyde nitrique synthétase endothéliale (eNOS), inductible (iNOS) et neuronale (nNOS) dans le thalamus médian, le colliculus inférieur et le cortex frontal (une région cérébrale non-atteinte) chez les rats où la déficience en thiamine fut induite par un régime déficient en thiamine et aussi par l'administration quotidienne de pyrithiamine, un antagoniste central de la thiamine.

Nous avons observé une augmentation significative des niveaux de l'ARN messager et de l'expression protéique de l'isoforme endothéliale (eNOS) proportionnelle à la sévérité de la dégénérescence neurologique et de la perte neuronale dans le thalamus médian et le colliculus inférieur.

Aucun changement dans l'expression des autres isoformes du NOS n'a été observé. Ces résultats suggèrent que l'endothélium vasculaire est un site important de production de NO dans les dans le cas de le cerveau des rats déficients en thiamine et que celui-ci pourrait être la cause des dommages aux structures vulnérables dans cette pathologie.

Mots-clés: Déficience en thiamine, Oxyde nitrique (NO), Endothélium vasculaire, Oxyde nitrique synthétase (NOS), Oxide nitrique synthétase endothéliale (eNOS), Oxide nitrique synthétase inductible (iNOS), Oxide nitrique synthétase neuronale (nNOS), Thalamus médian, Colliculus inférieur, Lésion oxydative, Encéphalopatie de Wernicke (EW), Système nerveux central (SNC).

Abstract

Thiamine deficiency is a cause of selective neuronal cell death in thalamic structures. Previous studies provide evidence for a role for nitric oxide (NO) in the pathogenesis of cell death due to thiamine deficiency. In order to ascertain the origin of the increased NO in the thiamine deficient brain, expression of the nitric oxide synthase isoforms endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) was measured in the medial thalamus and the inferior colliculus, and compared to the expression in the frontal cortex (a spared region in this particular pathology) of rats in which thiamine deficiency was induced by feeding of a thiamine-depleted diet and the daily administration of pyrithiamine, a central thiamine antagonist.

Endothelial NOS mRNA and protein expression were significantly increased as a function of the severity of neurological impairment and degree of neuronal cell loss in the medial thalamus and inferior colliculus. No significant alterations in the expression of other NOS isoforms were observed. These findings suggest that the vascular endothelium is a major site of NO production in the brain in thiamine deficiency and that eNOS–derived NO may account for the selective injury to histologically targeted regions in this disorder.

Keywords : Thiamine Deficiency, Nitric Oxide (NO), Vascular endothelium, Nitric oxide synthase (NOS), Endothelial nitric oxide synthase (eNOS), Inducible nitric oxide synthase (iNOS), Neuronal nitric oxide synthase (nNOS), Medial Thalamus, Inferior Colliculus, Oxidative damage, Wernicke Encephalopathy (WE), Central nervous system (CNS).

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Introduction

1-Sommaire

L'Encéphalopathie de Wernicke (EW) est une atteinte neurologique sérieuse causée par la déficience en thiamine (vitamine B1) et est souvent associée à un état général de malnutrition. Dans le monde occidental, on retrouve principalement cette pathologie chez les patients souffrant d'alcoolisme chronique, de vomissements associés à la grossesse sévères, d'autant que par les anorexiques et les sidéens. D'autre part, la consommation abusive d'alcool entraîne également des changements rendant ainsi les patients plus susceptibles à cette pathologie.

L'EW a été initialement décrite en 1881 chez trois patients souffrant d'ataxie, de nystagmus vertical et de changement cognitifs. Depuis lors, plusieurs chercheurs ont tenté de déterminer l'étiologie de cette pathologie. Il est maintenant établi qu'une insuffisance en thiamine est la cause principale de l'EW. Cependant, les mécanismes physiopathologiques menant à l'apparition de lésions histologiques dans certaines régions spécifiques du cerveau demeurent jusqu'à maintenant inconnus.

La déficience en thiamine induite par la pyrithiamine (DTP) chez le rat est un modèle qui émule l'EW en entraînant la dégénérescence sélective de certaines structures cérébrales. Chez le rat, la DTP évolue progressivement et récapitule les symptômes neurologiques et la neuropathologie de l'EW, soit l'apparition de lésions focales symétriquement distribuées (Troncoso et al., 1981).

La pyrithiamine est un analogue central de la thiamine qui franchit la barrière hémato-encéphalique et entraîne chez l'animal des symptômes neurologiques sévères (ataxie, opisthonus, nystagmus, perte du réflexe de redressement, convulsions et finalement le coma). La pyrithiamine s'accumule dans les structures cérébrales (Rindi et Perri, 1961; Rindi et al., 1963) et entraîne une diminution rapide des taux de thiamine cérébrale en inhibant la thiamine pyrophosphokinase (Gubler, 1968; Johnson et Gubler, 1968), l'enzyme

responsable de la conversion de la thiamine en ester diphosphate. De plus, la pyrithiamine inhibe la capture de la thiamine dans les tranches de cerveau (Nose et al., 1976) et peut déplacer la thiamine dans des préparations nerveuses (Cooper, 1968).

Le radical libre monoxyde d'azote (NO) possède des effets toxiques sur le système nerveux central (SNC) (Iadecola, 1997). Cette substance cause des dommages oxydatifs en réagissant avec les anions de superoxyde ($^{\cdot}O2^-$) pour former le péroxynitrite ($ONOO^-$), un puissant anion oxydant qui se est décompose rapidement en radical hydroxyle $^{\cdot}OH$, un oxydant hautement réactif qui réagit de façon non spécifique (Merrill et Murphy, 1996). Une insulte oxydative peut mener à la peroxidation des lipides, la décomposition de l'ADN et à l'inactivation des enzymes piégeuses de radicaux libres. Les producteurs de radicaux libres dans le SNC sont les mitochondries, la xanthine oxydase, le métabolisme de l'acide arachidonique et l'oxydation spontanée de molécules telles que les catécholamines et l'hémoglobine.

Les anions de superoxyde formés par diverses réactions sont normalement transformés en peroxyde d'hydrogène (H_2O_2) de façon spontanée ou aussi par les actions de la superoxyde dismutase (SOD). Une étude par Langlais et al. en 1997, a démontré la présence de substances oxygénées réactives dans le thalamus des animaux DTP. Jusqu'à maintenant la source exacte de la production excessive de substances oxygénées réactives dans la DTP n'était pas claire.

Les cellules endothéliales et les astrocytes sont des producteurs importants de NO (Murphy et al., 1990; Moncada et al., 1991). Il a été démontré que le NO peut endommager la BHE (Au et al., 1985). On sait aussi que les radicaux libres peuvent augmenter la perméabilité des cellules endothéliales (Chan et al., 1991). Il est donc possible que le NO puisse modifier l'intégrité fonctionnelle de ce type de cellule et ainsi jouer un rôle important dans la rupture de la BHE chez les animaux DTP (Calingasan et al., 1995a; Harata et Iwasaki, 1995).

Même lorsque l'activité totale de NOS (l'enzyme responsable de la production de NO), dans le cerveau est réduite dans les animaux traités à la pyrithiamine (Rao et al., 1996), l'expression de l'isoforme endothéliale est augmentée dans les régions vulnérablesdu cerveau ainsi que dans les vaisseaux sanguins.

De plus, le traitement de rats au deprenyl, un piégeur de radicaux libres, accroît la survie neuronale chez les rats DTP (Todd et Butterworth, 1998b), ce qui indique que les substances réactives oxygénées, dont le NO, jouent un rôle critique dans la pathogenèse des atteintes cérébrales induites par la déficience en thiamine.

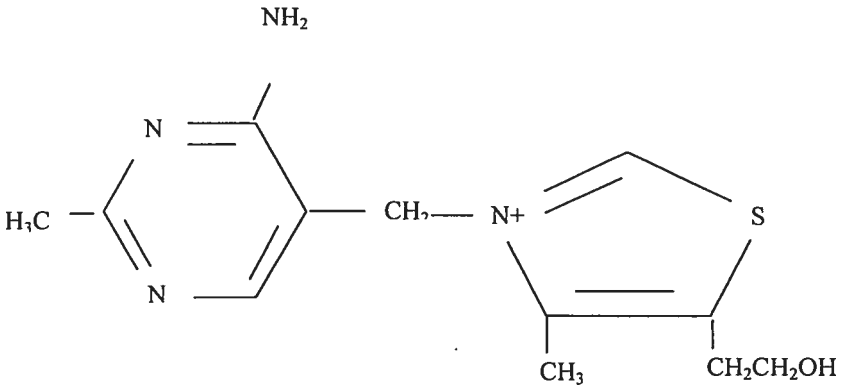
Dans une importante étude par Calingasan et al. (2000) le rôle de l'induction de eNOS par la voie dépendante de l' ICAM-1 est un facteur critique dans la mort neuronale causée par les dommages oxydatifs dans les neurones dont le métabolisme est compromis.

General Introduction

1-1 CHEMICAL STRUCTURE OF THIAMINE , SOURCES AND NEEDS

Thiamine, which is also known as vitamin B1 is a water-soluble vitamin whose structure contains a pyrimidine ring and a thiazol cycle bonded to a quaternary ammonium by a methylene bridge (Figure 1.1)

THIAMINE



PYRITHIAMINE

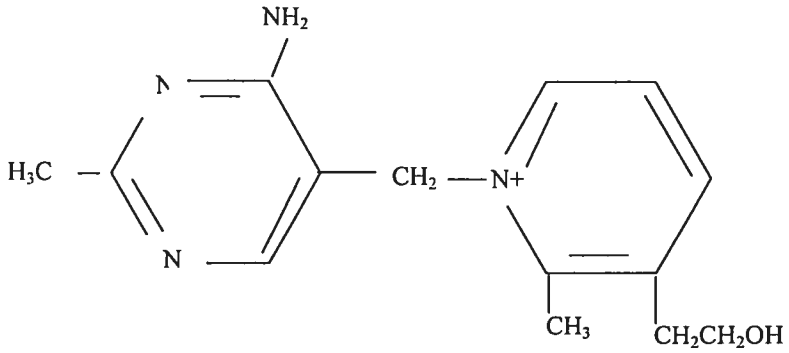


Figure1.1 Chemical structures of thiamine and pyrithiamine.

Thiamine is a vitamin essential to most mammals, since they are incapable of autonomous synthesis. The main sources of this nutrient are cereal products, vegetables, meat, fish, nuts and dairy (Robinson, 1966; Thiessen, 1978). The brain itself contains 1 μg/g of fresh tissue (Davis and Icke, 1983). Thiamine plays an extremely important role in glucose metabolism that takes place in order to fulfill cellular energetic processes; therefore the necessary intake to satisfy those needs must be adjusted according to need.

Theoretically, an average daily intake of approximately 1.0 and 1.5 mg per diem would be sufficient (Dietary Reference Intake or RDI, 2003).

In the brain, thiamine is most often encountered as thiamine diphosphate (TDP) (Spector, 1982; Davis and Icke, 1983) in contrast with the haematological pathway where the preferred form is that of thiamine monophosphate (TMP). This difference is indicative of the probability that thiamine transport in the central nervous system (CNS) is dependent on a phosphorylation step (Barchi and Braun, 1972; Nose and al, 1974; Spector, 1976).

The existence of a specific membrane phosphatase that is favorable to transport would explain the reason why certain regions of the brain would be less vulnerable to the depletion of thiamine stores than others.

1.2 ROLE OF THIAMINE IN THE CNS

Thiamine plays its most significant role in the CNS as an enzyme cofactor. There are three thiamine-dependant enzymes in the CNS: Pyruvate Dehydrogenase Complex (PDHC), α-Ketoglutarate Dehydrogenase (αKGDH) and Transketolase (TK).These three enzymes are critical factors in glucose metabolism.

1.3 CLINICAL IMPLICATIONS OF THIAMINE DEFICIENCY.

1.3.1 Wernicke –Korsakoff Syndrome

Wernicke encephalopathy part of the Wernicke-Korsakoff syndrome, is a neurological disorder whose aetiology is generalized thiamine deficiency, usually the product of poor nutritional status. In economically developed countries, the highest incidence is found amongst the chronic alcoholic patient population. Alcohol abuse in itself leads to dysfunction of thiamine absorption making these same individuals even more vulnerable to this vitamin deficiency.

While experimenting by feeding pigeons with a thiamine-free diet, Sir Rudolph Peters (Kinnersley and Peters, 1930) successfully demonstrated the accumulation of pyruvate and lactate at the brainstem that preceeded the appearance of neurological symptoms. This discovery was the first to suggest the existence of a « biochemical lesion ».

Later on, Bowman et al. (1939) described the therapeutic effect of thiamine administration in the treatment of a patient suffering from cognitive changes (which are one of the symptoms encountered in this pathology). Thiamine was used successfully to reverse the ophthalmological as well as cognitive symptoms by Joliffe et al. in 1941.

On the other hand, the residual psychosis, also known as Korsakoff psychosis, seems to be refractory to this treatment modality. Thus, treatment of Wernicke encephalopathy with thiamine has been standard for several decades but the pathophysiology of this disorder has not been entirely explored.

1.3.1.1 Clinical presentation

This syndrome can be described as having an acute stage, that of Wernicke encephalopathy which is sensitive to treatment with thiamine and the more chronic stage, that of Korsakoff psychosis which seems refractory to this treatment modality. Both stages are respectively the neurological and psychiatric facets of a same syndrome which can be found amongst affected patients.

Three types of symptoms such as cognitive deficits, ataxia and also the ophthalmological type of symptoms such as ophthalmoplegia or vertical nystagmus characterize Wernicke encephalopathy (EW). All of these symptoms are seldom found in conjunction, but combinations of some of them and most frequently, ataxia and ophthalmological manifestations are the most common clinical profile presented in practice.

The characteristic cognitive alterations seen in this syndrome is inclusive of a significant level of apathy, difficulty concentrating on a particular task or thought as well as amnesia and perceptional alterations. In a few cases, the reversal of these behavioural and cognitive symptoms is achieved upon therapeutic thiamine administration. In most patients, improvement of the symptoms is progressive and sometimes is replaced by learning disabilities and (short-term) memory abnormalities. Memory integrity is rarely recovered.

Ataxia which is an important symptom for an accurate diagnosis is harder to confirm clinically, since it can vary from a subtle motor coordination problem to a severe handicap and it is only after performing a specific neurological examination, that the diagnosis can be rendered. Thiamine is only marginally successful as a treatment for this symptom.

Ophthalmologic manifestations are important landmarks and can therefore assist in rendering differential diagnosis. These include nystagmus (85%), paresthesis of lateral

abductors (54%) and paresthesis of coordinated vision (44%). Ophthalmological symptoms are rapidly alleviated after therapeutic thiamine administration.

Korsakoff psychosis is a very unique and characteristic set of symptoms in this disease process and it presents with short-term memory problems and learning deficiencies. Approximately 20% of patients recover completely from these symptoms and an additional 20% of them is non-responsive to treatment, with most of the patients falling in between these extremes.

1.3.1.2 Neuropathology

In an important series of studies, Victor et al. (1989) showed after the post-mortem evaluation of the brains of WE patients, macroscopic histopathological changes that are inclusive of an increase in size of the third and lateral ventricles and the thalamic subventricular regions, which are affected by lesions. On the other hand, cerebral atrophy is found in 20% of the patients and most often seen in the frontal lobes (Harper, 1983).

Rapid and accurate diagnosis of the disease is effective not only towards promptly improving the neuropathological abnormalities. The selectively vulnerable regions do not have a common embryological source (Hamilton et al., 1952) and do not share vascularisation.

The earliest histological changes in thiamine deficiency are morphological alterations in glia (Collins, 1967; Robertson et al., 1968). Macroscopic atrophy of the mamillary bodies can be seen when the dissection of a fixed brain is performed. This lesion is symmetrical with pinpoint hemorrhages in some cases.

Lesions found in the gray matter are similar and can be observed around the aqueduct, inferior and superior colliculus and the inferior portion of the IVth ventricle. The cerebellar vermis is mainly targeted at the folia, anterosuperior to the primary fissure. In cases of both Korsakoff psychosis and WE the lesion distribution is similar, with the

exception that in the latter the additional implication of lesions in the medial thalamus and hypothalamus has been found. Other targeted regions are the thalamus; hypothalamus, midbrain, inferior olive, cerebellar vermis and the vestibular lateral nuclei (Torvik, 1985; Mair et al.1979; Victor, 1976) as well as the basal nucleus of Meynert (Arendt et al., 1983).

An interesting particularity of this pathology is that although the periventricular regions are the most affected by lesions, most of its neighboring structures are spared. This is a good example of the regional specificity of the lesions occurring in this nutritional disorder. At histological observation it is important to discern that the damage seen in the mammillary bodies and sub-ependymal area is principally caused by chronic degeneration of neuropil, which develops spongiosis, and loss of myelinated fibers. These types of lesions are due to severe endothelial inflammation but in this case, neurons are not especially targeted (Torvik, 1985; Victor, 1976).

Contrary to this, in the thalamus and inferior olive, the exact opposite type of lesions are found, so that neurons are particularly damaged, and relatively sparing the neuropil (Torvik, 1985; Victor, 1976). Torvik's 1985 study suggested the presence of two different types of lesions and indicates the possibility that cellular brain injury might be the product of several mechanisms.

Lesions to the cerebellar hemisphere and to the anterior vermis are characterized by the decrease in Purkinje cells and the augmentation in Bergman astrocytes that in more severe cases are accompanied by an important neuronal loss in the granular layer.

1.3.1.3 Thiamine status and alcoholism

In economically developed countries, the most frequent cause of thiamine deficiency is chronic alcohol abuse. This is caused by the generally poor nutritional status seen in these individuals and the insufficient thiamine consumed in their diets (Leevy et al.,

1965; Neville et al., 1968). To add to this insult, excessive alcohol consumption alters normal thiamine phosphorylation.

It has been shown in both humans, (Tomasulo et al., 1968; Thomson et al., 1970) as well as in a rat model (Hoyumpa et al., 1978), that ethanol reduces intestinal absorption of this substance. It was shown that following oral administration of 20mg of thiamine, maximal absorption is of 4.77±0.36mg in normal patients but on the other hand, the absorption is decreased to 1.50±0.3mg in alcoholic patients.

The mechanism proposed to explain this phenomenon was the inhibition of active transport (Hoyumpa et al., 1975) through suppression of Na^+K^+ATPase in the enterocytic basolateral membranes (Hoyumpa et al., 1977).

Intestinal concentration seems to be a determining factor for inefficient absorption more so than the length of time of the exposure of the enteric tissues (Hoyumpa et al., 1978). Another aggravating factor is the common co-deficiency in alcoholics of vitamins B_6 and B_{12}. This leads to a reduction in thiamine absorption (Thomson et al., 1972; Howard et al., 1974; Nishino and Itokawa, 1977). Adding folates to the diet improves this effect (Davis and Smith, 1974).

Alcohol abuse also reduces the stores and capture of thiamine in the liver and also increases the depletion of this substance (Heaton, 1977; Howard et al., 1974; Chi-Po Chen, 1978). The consequence is that in the alcoholic patient the depletion of the hepatic thiamine stock is more rapid and its replacement mechanism is less efficient.

Alcohol abuse slows down TPKase activity which catalyses the synthesis of TDP, the active form of thiamine when it is utilized as a cofactor (Davis et Icke, 1983; Leevy and Baker, 1968). This downregulation of the conversion rate could explain why WK patients are less responsive to thiamine treatment.

1.4 EXPERIMENTAL WERNICKE ENCEPHALOPATHY

1.4.1 Treatment with Pyrithiamine (PTD)

Pyrithiamine-induced thiamine deficiency or PTD, is a recognized experimental model that is used to reproduce and study the pathophysiological changes of the human version of this disease, which is Wernicke encephalopathy, because the biochemical changes and selective histological lesions seen in the human disease are closely mirrored in this animal model (Troncoso et al., 1981).

Experimental thiamine deficiency in rodents is an excellent means to study the different mechanisms through which a general and chronic dysfunction of oxidative metabolism can lead to selective neuronal loss. The targeted brain regions usually start showing alterations after 10 consecutive days of treatment, namely symmetrical lesions in the thalamus, mammillary bodies, the pons and the gray matter in the periaqueductal area. The cerebellum, cortex, and striatum are mostly spared. Lesions are characterized by severe inflammation and hemorrhagic necrosis that affects astrocytes, myelinated layer and dendrites. At the symptomatic stage (after the loss of the righting reflex), these lesions become more extensive and reach the thalamus, hippocampus, the inferior colliculus, the lateral vestibulary nuclei, the inferior olive, and the globus pallidus (Troncoso et al., 1981; Irle and Markowitsch, 1983).

Neuropathologically, the first change in the vulnerable regions is the disruption of the blood-brain barrier (BBB) (Calingasan et al., 1998). Cell death and increased immunoreactivity accumulation to ß amyloid precursor protein (APP) in perikaya and abnormal neurites around the lesions follow this (Calingasan et al., 1995). Mechanisms responsible for the region specific BBB disruption, the cellular loss, and the altered expression of APP in the thiamine deficient rat are so far not fully understood. A favored hypothesis is the one that proposes that certain

substances released by endothelial cells and microglia such as nitric oxide (NO) or other producers of free radicals are the likely sources of damage. Relatively recently, an increase in the production of free radical species was shown in thiamine deficiency (Langlais et al., 1997).

NOS (the subject of this study) plays an important role in CNS physiology but in some cases, it is also a powerful mediator for neurotoxicity in several of the pathologies that affect the central nervous system. Several prior studies in other neuropathologies, have proposed that the excessive production of NO can be related or even be the cause of the increased permeability of the BBB (Mulligan et al., 1991; Corbet et al., 1992; Boje, 1996). Microglia are involved in the pathogenesis of neuronal cell loss in certain neuropathologies such as Alzheimer's (AD) or Parkinson's (PD) Diseases (Barron, 1995). It has been demonstrated in *in vitro* models, that activated microglia damage neuronal cells through NO (Chao et al., 1992).

On the other hand, oxidative stress derived from mitochondrial dysfunction is also critical in the development of several pathologies including the aforementioned ones (AD and PD) as well as ischemia, Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) (reviewed by Fiskum et al., 1999). The majority of these diseases have shown several common mechanisms that contribute to the neurodegenerative process interestingly and among others, inhibition of α ketoglutarate dehydrogenase (αKGDH) which is a thiamine-dependent enzyme in the TCA cycle that is affected by several factors (revised by Gibson et al. 2000).

Thiamine deficiency leads to oxidative mitochondrial dysfunction and thus to neuronal death (Héroux and Butterworth, 1992). Mitochondrial dysfunction can be reversible after thiamine cofactor administration (Sparacia et al., 1999).

This inhibition of αKGDH is the earliest metabolic pathological event seen (Butterworth et al., 1986, 1989). It leads to alteration in mitochondrial function which is conducive to a cascade of abnormalities that end in oxidative damage.

In the PTD rats, this damage is inclusive of the factors mentioned earlier such as heightened BBB permeability, augmentation in free radical production and increase in the expression of superoxide dismutase (SOD) (Todd and Butterworth, 1997) which is accompanied by increased microglial activity (Todd and Butterworth, 1999). Other important effects are increased production of NO (Calingasan et al., 1998) and glutamate excitotoxicity caused by the increase in extracellular glutamate concentrations and the impaired function of the glutamate transporters (Hazell et al., 1993, 2001).

1.4.1.2 The PTD Rat Protocol

The animals used in this particular study, were male Sprague-Dawley rats weighting approximately between 200 and 225 g. They were weighed daily and were given a subcutaneous (sc.) injection of pyrithiamine (0.5mg/kg). Rats in the control group were pair fed to the ones in the PTD ("symptomatic") group and fed the same amount of thiamine deficient food. The control animals also received a supplemental sc. injection of thiamine (0.1 mg/kg).

After 12 days of continual treatment, an additional group was designated amongst the PTD-treated rats and selected for a "pre-symptomatic" group after careful observation and evaluation for characteristic symptoms and signs such as development of rotational movement and tendency towards backwards movement without the presence of seizures or loss of the righting reflex (Hazell et al., 1998a). The rats in this group were sacrificed at this time.

Treatment was continued in the rest of the groups, as previously described, until they displayed loss of righting reflex, that is, when they were unable to turn over when placed on their backs. This was considered as the acute symptomatic stage.

1.4.1.3 Thiamine Levels

Thiamine deficiency is generalized and systemic. The brain is the last organ to be affected, which is evidence of how this structure is particularly protected.

Neurological damage is only apparent when thiamine serum levels reach 20% of their normal values (Aikawa et al., 1984). Neurological symptoms are not manifested until TDP decreases in the brain (Pincus and Wells, 1972). In order for the neurological repercussions to appear, thiamine levels must reach approximately 20 to 50% of their control value in the brain, (McCandless and Schenker, 1968; Pincus and Wells, 1972; Aikawa et al., 1984; McCandless, 1985). After examination of the different regions, the pons and midbrain showed a significant decline in the TDP levels with regard to the cerebellum and cortex which are relatively spared (Pincus and Wells, 1972; Pincus and Grove, 1970). It has also been demonstrated that in asymptomatic animals the TDP level was around 35% of its control value but in those animals that were symptomatic this value was only of 25%.

In 1973, Murdock and Gubler showed that the use of pyrithiamine was significantly more effective in producing thiamine deficiency than strictly dietary deficiency. It has been also shown that if neurological manifestations become noticeable when brain thiamine level attains 20%, these symptoms are easily reversed after a minimal increase of this nutrient (26%) (McCandless and Shenker, 1968).

1.4.1.3 Cerebral energy metabolism

Previous studies have shown that cerebral vulnerability is related to the decrease in adenosine triphosphate (ATP) levels in the regions that are targeted by histological lesions (Aikawa et al., 1984). Since αKGDH is a key rate limiting enzyme of the ATP-yielding TCA cycle, specific reductions in its activity (Gibson et al., 1984; Butterworth et al., 1986; Butterworth and Héroux, 1989) are probably responsible for the decline in the total energy production and by this means of the decrease in ATP in the sensitive regions of the brain during PTD. This likely is a significant contributor in determining the survival of cells in particular brain regions during the course of the protocol.

PTD leads to a decline in generalized glucose utilization. A localized rise, then a decline follows this initial decrease, and this phenomenon is limited to the histologically targeted regions (Hakim and Pappius, 1983). The alterations in glucose utilization precede the appearance of the neurological symptoms. As demonstrated by Aikawa et al. in 1984, the fall in ATP and phosphocreatine (PCr) levels is probably linked to the final decrease in glucose utilization. Administration of thiamine after these developments, is only partially effective in restoring normal glucose utilization and it has been shown that this effect is improved if administration of thiamine takes place prior to the temporary increase stage (Hakim et al., 1983). These types of studies suggest that the histologically targeted regions in thiamine deficiency probably develop functional impairments before the acute symptomatic stage occurs.

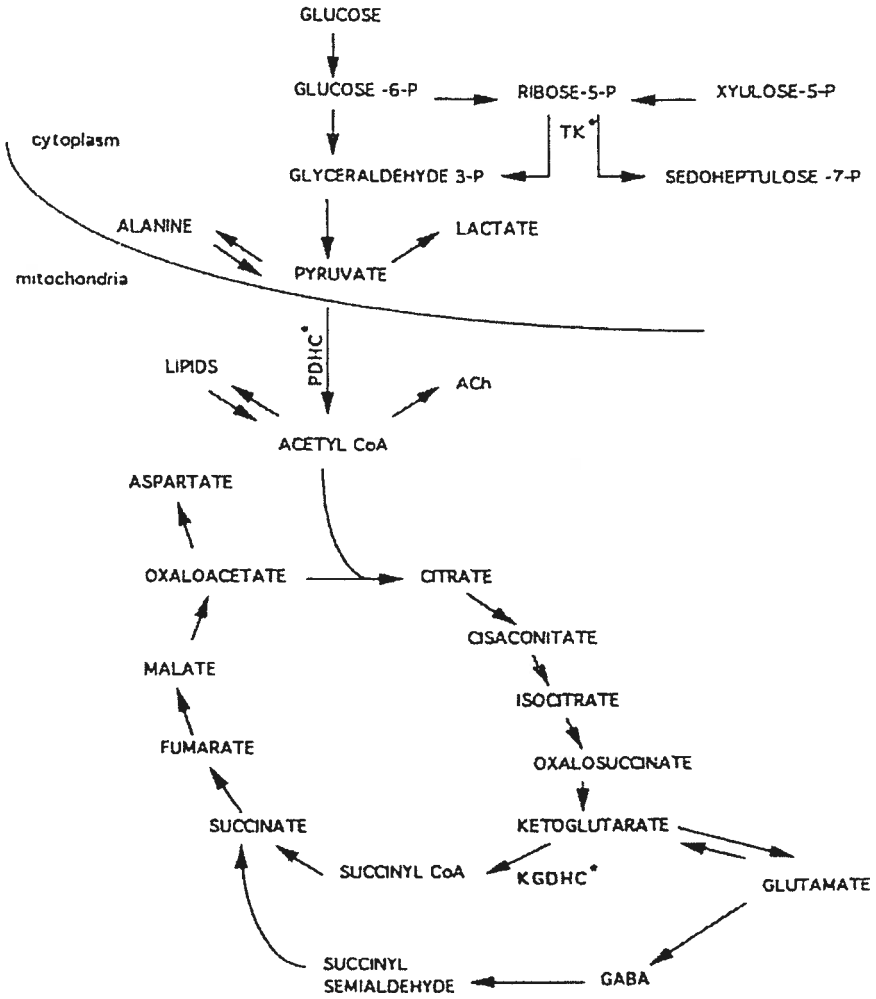
1.5 The « Biochemical »Lesion.

As it was stated in the general introduction, three major enzymes depend on thiamine in the form of TDP for their function. These are PDHC, αKGDH and TK.

In early studies by Peters (1936 and 1969), thiamine deficit brought about by a diet based on polished rice, led to the occurrence of opistonus and accumulation of lactate in brainstem structures in a pigeon model that occurred prior to the development of the structural lesions. These metabolic abnormalities which were reversed *in vitro* gave rise to the development of the concept of a "biochemical lesion" caused by abnormal pyruvate oxidation.

In more recent studies, it has been shown that this lesion is the product of a decline in the activity of brain αKGDH that occurs during PTD treatment in rats (Gibson et al., 1984; Butterworth et al., 1986). Also, the fall in the activity of αKGDH in the symptomatic PTD rat brain has been associated with an increase in alanine (Gibson et al., 1984; Butterworth and Héroux, 1989) and lactate (McCandless, 1982; Hakim, 1984; Munujos et al., 1993), supporting the hypothesis that suggests that there is a decrease in pyruvate entering the tricarboxylic cycle flux (TCA). αKGDH activity is severely restricted in most regions that eventually develop histological lesions in PTD animals (Gibson et al., 1984; Butterworth et al., 1986; Butterworth and Héroux, 1989) particularly, in the medial thalamus.

The reduction in total αKGDH activity is thought to be the cause for the reversible reduction in the concentrations of neuroactive aminoacids such as: glutamate, aspartate, and GABA, which demonstrates the importance that the activity of these amino acids may have on the formation of this biochemical lesion. Despite this, normalization of αKGDH, GABA and aspartate activities after thiamine replenishment is only partial which might be a sign of the permanent structural damage that takes place in this neuroanatomical region.



1.3 Schematic diagram of the glycolytic and TCA pathways. Thiamine dependent enzymes are indicated with an asterisk. (Adapted from Hazell et al., 1998a).

Although the changes in pyruvate and lactate levels would suggest that there could possibly exist a reduction in PDHC levels, these remain unperturbed during PTD (Gibson et al., 1984; Butterworth et al., 1985; Elnageh and Gaitonde, 1988).

But dramatic and often permanent decreases have been observed in transketolase (TK) levels in both vulnerable and refractory regions to the characteristic histological lesions in the brain (McCandless and Shenker, 1968; McCandless et al., 1976; Gibson et al., 1984; Giguère and Butterworth, 1987) (Figure 1.3). Moreover, progression towards the manifestation of neurological symptoms has been correlated to reduced levels in TK activity in the brain (Dreyfus, 1962,1967). TK plays a role in the pentose phosphate shunt, which is a pathway that produces both ribose sugar residues needed for nucleic acid synthesis, and fatty acids necessary for the adequate sustenance of the membrane phospholipid integrity.

It was shown in 1996, that destabilization of TK protein or decrease in mRNA translation of this enzyme produced by thiamine deficiency, is the principal cause for the decrease in activity (Sheu et al., 1996). On the other hand, despite the reduction in TK activity, the total activity of the pentose phosphate shunt seems undisturbed in PTD-treated animals (McCandless et al., 1976). This leads to the notion that this pathway may well be an important metabolic store. With the evidence accumulated so far on the subject, the conclusion can be drawn that modifications in PDHC and TK activity levels are probably not significant factors in the development of the reversible neurological symptoms of PTD. In contrast to this, a prolonged period of reduced TK activity is likely to lead to insufficient production of nucleic acids, lack of integrity of the cellular membranes and the ensuing demyelination that is a characteristic of WE.

In studies made in 1940 by Mann and Quastel on the development of the neurological symptoms that accompany thiamine deficiency, it was shown that the rate of

synthesis of acetylcholine was significantly elevated in brain slices of thiamine deficient pigeons after *in vitro* addition of thiamine. More recently, it was reported that thiamine deficiency is a cause for the decline in levels of the precursor for acetylcholine, Acetyl-coenzyme A (Heinrich et al., 1973) in the brain, and logically those of acetylcholine in the same organ (Lissak et al., 1943; Cheney et al., 1969; Heinrich et al., 1973). These findings were contested since other research teams were not able to reproduce these results and found no alterations in brain acetylcholine metabolism in the same model (Reynolds and Blass, 1975,Vorhees et al., 1977). In support of this point of view, the activities of a cholinergic marker, the enzyme choline acetyltransferase remained stable throughout the duration of the treatment (Thompson and McGeer, 1985; Amstrong –James et al., 1988) which means that there is no significant loss of neurons in this model. Furthermore, administration of eserine, which is an acetylcholinesterase inhibitor, before the onset of seizures, increases PTD rat survival time (Cheney et al., 1969). Moreover, after observation of the effects of cholinergic agents on the ability of thiamine deficient animals to perform a string test test in which they cross the length of a string, and also on open field staring behaviour, it has been established that a central muscarinic cholinergic lesion is develops early in the pathology (Gibson et al., 1982).

After gathering the existing evidence, it can be concluded that there are dysfunctions in the cholinergic system in thiamine deficiency yet these are more likely to be due to diminished acetylcholine synthesis than to a structural lesion (Gibson et al., 1984).

1.5.1 Effects on thiamine-dependent enzymes during PTD.

As stated in the previous section, pyrithiamine induced thiamine deficiency has distinct and specific effects on thiamine-dependent enzymes that play a major role in cell energy metabolism.

Their obvious decrease when there is a general depletion of the cofactor, leads to decreased efficiency of the TCA cycle and consequently of lipid, acetylcholine, ATP and associated neurotransmitter synthesis and logically to CNS pathology (Dreyfus and Hauser, 1965; McCandless and Shenker, 1968; Hollowach et al., 1968; Butterworth, 1985b).

1.5.1.1 PDHC (Pyruvate dehydrogenase complex)

PTD treatment produces an increase in the levels of pyruvate and lactate. This is an effect that is particularly evident in the thalamus and vestibular lateral nuclei and sparing the cerebellum and cerebral cortex (Hollowach et al., 1968; Collins and Converse, 1970). In PTD, there is a decline in pyruvate oxidation (Bennett et al., 1966; Gubler, 1961). A few studies have found changes in the activity of this enzyme in the brain.

In 1968, Hollowach et al. observed a 25% decrease in this enzyme and proposed that this change could be the cause of the neurological alterations that develop in this pathology. Gibson et al. (1985) only found a significant reduction (32%) in the vulnerable regions (mammillary bodies) but it remained unchanged in the cortex. In that same year, it was shown that PDHC activities are selectively downregulated from 15 to 30% in the midbrain and pons (vestibular lateral nuclei). Thiamine administration reversed the characteristic neurological symptoms and simultaneously diminished the abnormalities in brain PDHC, but this was only true in the case of purely dietary thiamine deficiency. In the case of PTD, although the pathology is significantly accelerated, selective regional modifications in PDHC activity are not present (Butterworth et al., 1985).

1.5.1.2 αKetoglutarate Dehydrogenase (αKGDH)

PTD treatment in the rat has much more serious consequences and more extensive neurological repercussions than those caused by purely dietary thiamine deficiency. In PTD, αKGDH activities are substantially reduced in all areas of the brain with the most dramatic alterations occurring in the sites most vulnerable to pyrithiamine treatment (lateral vestibular nuclei, midbrain, hypothalamus, medulla and pons).

In some cases changes in αKGDH precede the onset of neuropathological symptoms. These reductions in αKGDH may explain prior observations of localized selective changes in energy metabolism and decreases in synthesis of glucose-derived neurotransmitters (acetylcholine, GABA, glutamate) in PTD animals.

After thiamine administration to PTD animals, characteristic symptoms are eliminated and the activity of αKGDH is restored in the brain. These observations indicate that the reversible symptoms of WE in humans are likely to be caused by deficient αKGDH function in the vulnerable regions of the CNS (Butterworth et al., 1986).

1.5.1.3 Transketolase (TK)

In PTD, total transketolase (TK) activities are always reduced in all of the structures observed (McCandless et al., 1976; Gibson et al., 1984). The activity of this enzyme is significantly reduced before the onset of neurological symptoms and the appearance of the symptoms is mirrored by an even stronger decline of TK.

In this model, transketolase activities are decreased equally in both vulnerable and refractory regions and these modifications remain unchanged after reversal of the neurological symptoms with therapeutic thiamine treatment (Giguère et al., 1987).

1.5.1.4 ATP Level

Some studies undertaken in the 1960's implicate the decrease in ATP levels in the development of necrotic oedematous lesions that are seen early in the pathogenesis and suggest that the fall in PDHC activity might be cause of this event (Robertson et al., 1968). On the other hand, a consistent level of ATP has been observed despite the decrease in PDHC, concluding that PDHC affects the diseases process by other means than those related to ATP production (McCandless et al.1968, 1976).

More recently, studies done in the PTD model (Aikawa et al., 1984) have shown that there are decreases in ATP and phosphocreatine (PCr), an energetically rich phosphate in the brainstem and the diencephalon and as expected that the cortex and cerebellum are characteristically spared. These declines become more severe after the onset of neurological symptoms. A few years later, these observations were confirmed but the same types of lesions were found also in the cerebral cortex (Takahashi et al., 1988).

These abnormalities in mitochondrial metabolism in thiamine deficiency are a product of the reduction in the synthesis of the thiamine-dependent enzymes which leads to a lowered ATP production. This is one possible source of the irreversible neurological changes in this model and in WE, which was also observed in a study by Parker et al. (1984).

1.5.1.5 Lactic Acidosis

Lactate accumulation is a well-known feature of thiamine deficiency (Kinnersley and Peters, 1930; Holowach et al., 1968, McCandless and Schenker, 1968). The

augmentation in lactate levels and the consequent acidosis this provokes, have been shown to be limited to the regions that later develop characteristic histological damage (McCandless, 1982; Hakim, 1984; Munujos et al., 1993).

In the rat model this causes changes in pH (acidification) in both in isolated cells and tissues (Mutch and Hansen, 1984). This is also an important factor in associated syndromes such as hypoxic and ischemic cerebral lesions (Myers, 1979; Pulsinelli et al., 1982; Plum, 1983; Yoshida et al., 1985).

In related pathologies, oedema has been shown, particularly in glial cells (Myers, 1979; Kalimo et al., 1981; Jenkins et al., 1984; Siesjö, 1985) and oedema can be produced by acidosis inside and well as in the exterior cellular environment (Collins, 1967; Robertson et al., 1968; Watanabe and Kanabe, 1978; Watanabe et al., 1981). Lactate accumulation and the change in pH that take place are probably factors contributing to neuronal cell death in both this model and WE (Hakim, 1984).

1.6 MECHANISMS OF NEURONAL CELL DEATH IN THIAMINE DEFICIENCY.

1.6.1 NMDA-receptor mediated excitotoxicity

Alterations in glutamate homeostasis are often suggested as being the sources of the pathology that develops in the CNS in the PTD animal. Glucose levels are decreased in the totality of the brain (Butterworth, 1982), and also in the characteristically vulnerable regions such as the thalamus and the pons (Butterworth and Héroux, 1989). These observations support studies that describe a decrease in the conversion of glucose radioactively labeled with [^{14}C] into glutamate in PTD rats (Gaitonde et al., 1975). Ca^{2+}

dependent glutamate release was also shown to be reduced in hippocampal slices of symtomatic animals (Lê et al., 1991).

During observations carried out in 1988 by Amstrong-James et al. it was recorded that progression and general aspect of damage in lesions to the medial thalamus which present during thiamine deficiency, have a similar appearance to those lesions present when excitatory aminoacids are administered The ultrastructural aspect of the affected thalamus is also similar to that observed in necrosis mediated by excitotoxicity (Olney, 1978). To support this finding, it was shown that it is possible to limit the damage caused by cell death in the vulnerable regions in this pathology using MK-801 which is a non-competitive antagonist of the NMDA receptor (Langlais and Mair, 1990; Todd and Butterworth, 1998a). Furthermore, another supporting factor that points to glutamate excitotoxic mechanisms is that extracellular glutamate concentrations were found to be augmented in the posterior thalamus during PTD (Hazell et al., 1993; Langlais and Zhang, 1993). Significant neuronal loss has been observed and it becomes apparent before the onset of neurological symptoms (Leong et al., 1994; Todd and Butterworth, 1998a; Hazell et al., 1998a).

Increases in the extracellular glutamate concentration in the vulnerable regions such as the thalamus in PTD animals can lead to unrestricted entry of Ca^{2+} into the cell. The loss of calcium homeostasis has been shown to be an important cause of cell death through excitotoxic processes in several of the neurodegenerative pathologies (Olney et al., 1971; Schanne et al., 1979; Farber et al., 1981; Raichle, 1983; Siesjö and Bengtsson, 1989).

Depolarization caused by glutamate can lead to the activation of both types of calcium channels: those that are NMDA-receptor mediated and also voltage sensitive calcium channels (VSCCs). This in turn can result in an excessive influx of Ca^{2+} into neurons. In a study published in 1998, *in vivo* binding of [^{3}H] nimopidine was analyzed in

PTD animals (Hazell et al., 1998b). This ligand is an antagonist of VSCCs that specifically binds to brain regions touched by ischemic insult which are likely to develop infarction (Hakim and Hogan, 1991). An increase in specific binding of [^{3}H] nimopidine was seen in the thalamus while binding in other regions is mostly non-specific. These modifications are seen before the development of significant cell necrosis and subsequent to the loss of the righting reflex, and this is probably evidence for depolarization and activation of the L-type of VSCCs in the thalamus. This may indicate the likelihood that an excitotoxic mechanism is involved in lesions developed in this neuroanatomical region.

Some observations suggest that the elevation in extracellular glutamate in the thalamus of acute symptomatic animals can result from a Ca^{2+} independant process (Hazell and Hakim, 1994). These findings support the hypothesis that the increase in extracellular glutamate levels seen in the thalamic region of symptomatic PTD animals, is due to a decrease in transmitter glutamate release and a rise in the non-vesicular component efflux from cytoplasmic reservoirs.

1.6.1.2 Selective reduction in astrocytic glutamate transporters

Increases in extracellular glutamate concentrations have been observed, particularly in the thalamus, one of the targeted regions in the PTD model. Despite this, in a recent study, a decrease in the protein expression levels of the astrocytic glutamate transporters GLT-1 and GLAST was shown *in vivo* (Hazell et al., 2001).

It was described a few years ago, that in the medial thalamus, apoptotic processes occur prior to the onset of symptoms in this region (Matsushima et al., 1997) or to the increase in extracellular glutamate levels (Todd and Butterworth, 1998). Later, the thalamus develops significant histological lesions that are characterized by pannecrosis, which might be indicative of an excitotoxic process. The proposed mechanisms for the

increase of this neurotransmitter include an elevation of excitation of glutamatergic neurons possessing NMDA receptors (Langlais and Mair, 1990), a decline in glutamate reuptake caused by depolarization (Hazell et al., 1998b), an increase in calcium independent glutamate release (Hazell et al.,2001) and dysfunction of the glutamate transporters.

In PTD rats at the acute symptomatic stage, a selective increase in extracellular concentration levels has been shown in the thalamus (Hazell et al., 1993; Langlais and Zhang, 1993). The fall in GLT-1 and GLAST is not apparent in pre-symptomatic animals at the 12^{th} day of treatment, which is concordant with prior observations that confirmed normal glutamate levels were maintained until the onset of the symptomatic stage (Hazell et al., 1993) and this supports the hypotheses that states that this decrease is a late event in this model. The results of these studies indicate that PTD leads to the loss of a critical astrocytic function, namely the maintenance of homeostasis of extracellular glutamate levels. This is caused by a malfunction of the transport of this neurotransmitter derived from the decline of GLT-1 and GLAST.

Glutamate transporter activity is also modulated by protein kinase C (PKC) and cyclic AMP (cAMP). Therefore alterations in phophorylation of the transporter and in cAMP levels could also lead to the downregulation of GLT-1 and GLAST protein expression. Another factor that might affect this expression, is the increase in reactive oxygen species (ROS) and induction of nitric oxyde synthase (NOS), which is an important souce of free radical production in the thalamus of PTD rats (Langlais et al., 1997; Calingasan et al., 1998). The increase in production of free radicals can also be a causal agent of the downregulation in glutamate transporters (Gegelashvili and Schousboe, 1997).

1.7 The Blood Brain Barrier (BBB)

It was suggested several decades ago, that loss of blood-brain barrier integrity might be a source of the brain lesions encountered in WE. Since then, a few studies have focused on the study of the integrity of this neuroptotective mechanism, particularly in the purely dietary experimental model and have reported changes in BBB permeability (Warnock and Burkhalter, 1968; Robertson and Manz, 1971; Manz and Robertson, 1972; Phillips and Cragg, 1984). Studies done in a more direct manner in the PTD model, utilizing immunohistochemical techniques, have shown disruption of the BBB that allows for passage of large molecules such as IgG or albumin before and during the onset of the symptomatic stage (Harata and Iwasaki, 1995; Calingasan et al., 1995a). Furthermore, the haemorraghic lesions classically seen in the vulnerable regions (Witt, 1985; Vortmeyer and Colmant, 1988; Calingasan et al., 1995a), rather indicate that loss of integrity of the capillary vessel walls takes place in PTD. Analogous findings have been reported in relation to the brains of WE patients (Schroth et al., 1991).

Pathophysiological processes may produce alterations in BBB integrity in PTD. The optimal function of the BBB is principally sustained by the cerebral endothelium. The low permeability characteristic of the BBB, is possible because of the presence in endothelial cells of tight junctions and limited vesicular profiles (Reese and Karnovsky, 1967; Brightman, 1977).

Mechanical opening of endothelial tight junctions are an unusual finding in the brains of PTD animals. When this structure has been observed, tight junction integrity has been described (Calingasan et al., 1995a; Watanabe, 1978). The augmentation in vesicular transport across endothelial cells seen in thiamine deficiency (Manz and Robertson, 1972; Calingasan et al., 1995a) has also been observed in other neuropathologies (Hirano et al., 1994).

On the other hand, prior studies demonstrating increased transport of high molecular weight molecules through the BBB have linked this phenomenon to augmented BBB permeability and also to cerebral oedema (Joó, 1971; Westergaard and Brightman, 1973). These particular studies suggest that the enhanced immunostaining seen in the cases of IgG and albumin in the PTD, are evidence of the likely access of detrimental products imported via the bloodstream into the brain parenchyma. An increase in BBB permeability has been shown as early as the 10^{th} day of pyrithiamine treatment and this trend continues as the pathology progresses towards the symptomatic stage (Hazell and Butterworth, 1997).

Although it is possible that the focal lesions seen in this model might be a product of loss of function of the BBB, this remains somewhat controversial. Some of the structures that are characteristically targeted in this model, such as the inferior colliculus, present a haemorraghic type lesion, in contrast to the ischemic type lesion seen in the thalamus (Vortmeyer and Colmant, 1978). Studies examining the brains of WE patients and those of PTD animals confirm that the thalamus is affected in a different manner than other targeted neuroanatomical structures in this particular pathology (Torvik, 1985; Matsushima et al., 1997; Hazell et al., 1998a).

Another possibility that has been explored in regards to BBB vulnerability is the one that suggests that this effect might be caused by a biochemically-mediated process. Recent studies have shown the presence of amyloid precursor protein (APP) in the targeted regions (thalamus and inferior colliculus) (Calingasan et al., 1995a, 1996). Studies recently undertaken, have demonstrated that the β amyloid peptide (Aβ), which has consistently been shown to form accumulations in numerous other neurodegenerative pathologies such as Alzheimer's (AD), Parkinson's (PD), Creutzfeld-Jakob (CJD), Huntington's (HD) and Down's syndrome (DS), and that is a fragmentation of APP, significantly increases the

fluidity of the neuronal membrane as well as contributes to lipid peroxidation (Avdulov et al., 1997)

These modifications in the membrane's lipid environment could be influential towards causing the BBB deterioration seen in PTD. Furthermore, Aβ has been shown to enhance endothelial cell permeability to albumin (Blanc et al., 1997). This might be a reason for the easier access of albumin across the brain parenchyma observed in this model (Harata and Iwasaki, 1995). This effect has been shown to be reversible when antioxidants were administrated (Blanc et al., 1997). Other studies have also reported that Aβ might be a possible cause of excitotoxic damage (Koh et al., 1990; Yanker et al., 1990; Mattson et al., 1992) and can stimulate apoptotic processes (Forloni et al., 1993; Loo et al., 1993). These results indicate that this substance contributes to the increased BBB permeability and the cell death encountered in PTD.

1.8 Oxidative Stress

The free radical nitric oxide ($^{\cdot}NO$) is known for having neurotoxic effects on the CNS (Iadecola, 1997). $^{\cdot}NO$ causes oxidative damage when it reacts with superoxide anions ($^{\cdot}O_2^-$) which leads to the formation of peroxinitrite ($ONOO^-$) which is a potent oxidative anion that reacts non-specifically (Merrill et Murphy, 1996). Oxidative damage can cause lipid peroxidation, DNA fragmentation and enzyme inactivation, including free radical scavengers. There are several sources of superoxide in the CNS and amongst these are mitochondria, xanthine oxidase, and metabolism of arachidonic acid and also oxidation of substances such as catecholamines and hemoglobin.

Under normal conditions, superoxide anions derived from various sources are transformed into hydrogen peroxide (H_2O_2) spontaneously, or catalysed by superoxide dismutase (SOD). Langlais et al. in 1997 described the presence of reactive oxygen species

in the thalamus of PTD-treated animals. The possible cause for the increase in free radical production in the case of thiamine deficiency is until now not fully understood but amongst the probable culprits that have been suggested are increased extracellular glutamate concentration levels and activation of NMDA receptors, alterations of the BBB, and activation of microglia and astrocytes. Activated microglia, as was shown in a recent study (Todd and Butterworth, 1998c), may be responsible for the enhanced production of free radicals. Reactive microglia are also characteristic of WE, presenting the possibility that free radical production might be a component of the human pathophysiology of this disease.

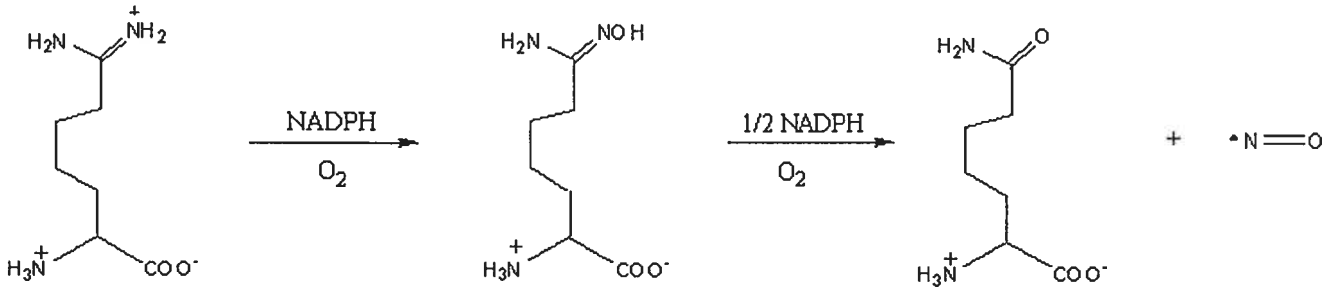
Augmented endothelial transport has been suggested as being a contributing factor to the dysfunction of the BBB seen in PTD (Calingasan et al., 1995a; Harata and Iwasaki, 1995). This indicates that the function of this type of cells might be compromised in this case. NO can penetrate the BBB (Au et al., 1985). Endothelial cells and astrocytes are known to be sources of NO production (Murphy et al., 1990; Moncada et al., 1991). Also free radicals can enhance permeability of endothelial cells in the brain (Chan et al., 1984). Therefore this free radical is likely to contribute to BBB disruption in PTD.

Despite a finding that describes a decline in total NOS activity in PTD animals (Rao et al., 1996) it has been shown in several studies including the present one that there is a significant increase in the endothelial (eNOS, NOS III) isoform of this enzyme in the classically targeted regions of the brain both by immunohistochemical methods (Calingasan et al., 1998) or as will be discussed later, by the increases observed in both protein and mRNA expression.

1.8.1 Nitric Oxide Synthase and NO

Nitric Oxide (NO) is a small, reactive molecule that is an important bioregulator. NO is used for a number of cellular signaling functions including blood vessel dilation, neuronal signal transmission, cytotoxicity against pathogens and tumours, coordination of heart rhythm and the regulation of cell respiration activity (Murad, 1999). NO is produced by the heme-containing metalloenzyme, nitric oxide synthase (NOS) (Fig.1.4). There are three NOS isoforms, of which two are thought to be constitutive (nNOS and eNOS) in nature and one which is inducible (iNOS) (Marletta, 1993). (Table 1.0)

Endothelial NOS (eNOS) is mostly membrane bound and formed only in endothelial cells (Furchgott, 1999). Neuronal NOS (nNOS), was identified in the cytosol of central and peripheral neurons. NO derived from its constitutive isoforms acts as a physiological regulator by relaxing vascular smooth muscle or by functioning as a neurotransmitter. These isoforms produce small amounts of NO for short periods of time in a Ca^{2+}/Calmodulin dependent manner upon stimulation. Endothelial NOS with the endothelial cell acting as a transducer, releases NO continously in varying amounts to regulate blood-vessel tone and by the same means the blood flow and pressure. Large amounts of NO produced for a prolonged time can cause vasodilation and hypotension. On the other hand, insufficient NO prodution can be a cause of hypertension.



L-arginine N^{ω}-hydroxy-L-arginine L-citrulline

Fig.1.4 NO production catalysed by Nitric Oxide Synthase

Characteristic	Endothelial NOS	Neuronal NOS	Inducible NOS
Dependency on Ca^{2+}, Calmodulin	Yes	Yes	No
Molecular weight in kD	140	155	130
Chromosomal location	7q35-36	12q24.2	17q11.12
Producing cells	Endothelial	Neurons	Macrophages,monocytes, astrocytes
Inductors of biosynthesis	No	No	Lipopolysaccharide (LPS), Inflammatory cytokines.
Inhibition by L-arginine analogs.	Yes	Yes	Yes
Inhibition by glucocorticoids	No	No	No

Table 1. Characteristics of human NOS isoforms

This suggests that NO is involved in the regulation of the vascular system (Ignarro, L., 1999). Within the CNS, NO is released in response to increases in intracellular Ca^{2+} that follow stimulation of glutamate receptors. The other function of NO, on which we focus this study relates to its neurotoxic effects since its excessive release is known to lead to cell death processes.

The third isoform of NOS is not present in resting cells but instead, the cells must be induced to express the enzyme, ergo the nomenclature inducible NOS (iNOS). Stimuli typically includes cytokines and lipopolysaccharide (LPS), and once expressed, the enzyme

generates copious amounts of NO. A number of cytokines are involved in the production of iNOS. Among them are IFN-γ, IL-1, IL-6, THF-α, GM-CSF (granulocyte-macrophage colony stimulatory factor) and PAF (platelet activating factor) exert the stimulatory effect whereas the suppression has been observed in the cases of IL-4, IL-8, IL-10, TGF-β (transforming growth factor), PDGF (platelet-derived growth factor) and MDF (macrophage deactivating factor).

$NO^{\cdot}$ may react with superoxide to form the highly toxic peroxynitrite anion:

$$NO^{\cdot} + {}^{\cdot}O_2^{-} \longrightarrow \mathbf{ONOO^{-}}$$

Which in turn may be transformed in an acidic environment to peroxynitrite and subsequently to the hydroxyl radical:

$$ONOO^{-} + H^{+} \longrightarrow \mathbf{ONOOH}$$

$$ONOOH \longrightarrow {}^{\cdot}\mathbf{OH} + NO_2 \longrightarrow NO^{-}_2 + H^{+}$$

Independent pathways are involved in the synthesis of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI).

The basis of the functional activity of NO is its dual action on some enzymes of target cells. The small amount of NO released by the constitutive isoforms is sufficient for the activation of the NO-sensitive enzymes (guanylate cyclase and ADP-ribosyl-transferase) that participate in NO signaling pathways. The larger amonts of NO generated by iNOS can also activate the NO-sensitive enzymes, but in several cell types the increased production of NO also exceeds the necessary concentration treshold to inhibit the action of

certain Fe+-containing enzymes, namely aconitase, NADPH-ubiquinone oxidoreductase, succinate-ubiquinone oxidoreductase, ribonucleotide reductase, NADPH oxidase and glyceraldehyde-3-phosphate dehydrogenase.

Activation of soluble guanylate cyclase by NO leads to the synthesis of cGMP, which in turn, causes the relaxation of vascular smooth muscle cells, inhibition of platelet adherence and aggregation, inhibition of neutrophil chemotaxis, and signal transduction in the central and peripheral nervous systems. NO causes autoribosylation of glyceraldehyde-3-phosphate dehydrogenase, which inactivates this glycolytic enzyme. NO also inhibits three mitochondrial enzymes: aconitase of the TCA cycle and NADPH ubiquinone oxidoreductase and succinate –ubiquinone oxidoreductase of the electron transport chain.

1.8.1.2 Nitric oxide effects in cell metabolism

NO is known to inhibit cytochrome C oxidase by binding to its heme group and leading to an upregulated generation of superoxide in the mitochondrial respiratory chain (Ghafourifar and Richter, 1997). In turn superoxide reacts with NO to yield peroxynitrite (Ghafourifar et al., 1999). NO or peroxynitrite block respiratory chain complexes I, III, and IV as well as the activity of cis-aconitase, an enzyme in the TCA cycle, by binding to the iron-sulfur centers. This inhibition leads to the truncation of the metabolism of acetyl coenzyme A to carbon dioxide, which is a crucial step in production of nicotinamide-adenine dinucleotide (NADH), which is necessary to fuel oxidative phosphorylation. These effects severely impair the cell's ability to conserve and manufacture an adequate reservoir of ATP.

On the other hand peroxynitrite decomposes into the hydoxyl radical (·OH) which is the most reactive species among those known to cause extensive cell and tissue damage in inflammation. It is widely known to cause lipid peroxydation, DNA mutations and protein modifications, and has apoptotic and cytotoxic effects on various cells. The other product

of the decomposition of peroxynitrite is the $NO_2^{\cdot}$ radical that causes nitration of the tyrosine residues of proteins.

NO is capable of inducing apoptosis depending on its concentation, flux and the cell type on which it is enacting. It activates the transduction pathways that lead to apoptosis. Pro-apoptotic effects are often observed when NO reacts with superoxide to yield the highly toxic peroxynitrite. Contrary to this, it also can protect cells against spontaneous or induced apoptosis. NO inactivates caspases through oxidation and S-nitrosylation of the active cystein, stimulation of cGMP-dependent protein kinase, modulation of Bcl-2/Bax family, induction of heat shock protein Hsp 70 and interaction with the ceramide pathway. The reduction-oxidation state of the cells appears to be determinant of the ultimate effects of NO on cell proliferation and survival.

1.8.1.3 Vascular Factors in Selective Neuronal Loss in PTD

A recent study by Calingasan et al. (2000) examines the importance and influence of oxidative stress and vascular changes that are seen in PTD. They approached this by testing the role of ICAM-1 which is an inducible cell-surface glycoprotein expressed in endothelial cells, leukocytes, macrophages and dendritic cells and that of eNOS which is the subject of the present study and is known to be influenced by inflammatory processes and therefore may lead to BBB degradation.

It has commonly been suggested that iNOS might be the culprit of cell death and damage in this particular neuropathology but it has been demonstrated in a prior study (Calingasan et al., 1998) that it is not necessary to induce the pathology cascade in PTD since deletion of the iNOS gene did not mitigate the subsequent and characteristic neurodegeneration or the induction of heme oxygenase 1 (HO-1), an indicator of oxidative

stress. Induction of HO-1, has been correlated to neuronal loss in the thalamic region in PTD (Calingasan et al., 1998).

In this study, transgenic mice missing the genes encoding ICAM-1 and eNOS were used, to demonstrate that both are critical factors in the selective neuronal loss of PTD.

The results showed that although eNOS is an effector of basal vasodilation, excessive endothelium-derived NO is likely to damage neurons.

The results obtained in the studies performed in the transgenic eNOS-null mice, suggest that the beneficial effects of vasodilation are less significant than the toxic effects it has on PTD. Indeed, the targeted disruption of the eNOS gene, blunted the deleterious effects on neurons typical of PTD. This is confirmatory evidence for the hypothesis that states that NO produced by endothelial cells contributes to TD–induced neurodegeneration.

CHAPTER 2: OBJECTIVES OF THE STUDY

2.1 OBJECTIVES OF THE STUDY

There is a growing body of evidence that implicates excessive or inappropriate production of nitric oxide (NO) in disturbances of the the electron transport chain and also in the disruption of the BBB as was mentioned in the introduction.

This study seeked to explore temporal gene and protein alterations of the different NOS isoforms in the PTD model of neurodegeneration.

mRNA and protein expression of eNOS was determined by RT-PCR and Western Blot respectively in the medial thalamus, inferior colliculus and frontal cortex of PTD rats at the presymptomatic and acute symptomatic stages with respect to pair-fed controls treated following the protocol outlined in the introduction. Appropriately designed oligonucleotides were used in the RT-PCR experiments and monoclonal antibodies specific for each NOS isoform were used for the Western Blot protein expression studies. A confirmatory immunohistochemical study was also carried out, using paraffin embedded sections of PTD rat brains at the progressing stages of the pathology, using the same monoclonal antibodies.

Earlier studies in TD have reported increased immunorectivity to NOS in different cell types (Calingasan et al., 1998 and 1999), but this is the first study to report consistent alterations in gene expression in one of the NOS isoforms in TD.

The results obtained demonstrated a rise in eNOS mRNA and an increase in eNOS protein expression in the PTD model. This augmentation of eNOS is concordant with the results reported in prior immunohistochemical studies (Calingasan et al, 1998) that were confirmed by immunohistochemistry also in the present study and moreover, provide further information with respect to the changes in eNOS expression in the PTD model.

2.2 Increased brain endothelial nitric oxide synthase gene expression in thiamine deficiency: relationship to selective vulnerability .

Increased brain endothelial nitric oxide synthase gene expression in thiamine deficiency: relationship to selective vulnerability.

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Wernicke's Encephalopathy

Abstract

Thiamine deficiency results in selective neuronal cell death in thalamic structures. Previous studies provide evidence for a role implicating nitric oxide (NO) in the pathogenesis of cell death due to thiamine deficiency. In order to ascertain the origin of increased NO in the thiamine deficient brain, expression of endothelial nitric oxide synthase isoform, (eNOS), was measured in the medial thalamus and in the inferior colliculus and compared to the frontal cortex (a spared region) of rats in which thiamine deficiency was induced through a feeding protocol of thiamine-deficient diet concomitant with daily administration of pyrithiamine, a central thiamine antagonist. eNOS mRNA and protein expression were significantly increased as a function of the severity of neurological impairment and the degree of neuronal cell loss in the medial thalamus and in the inferior colliculus. These findings suggest that the vascular endothelium is a major site of NO production in the brain in thiamine deficiency and that eNOS-derived NO could account for the selective damage to the thalamic structures as well as for those seen in the inferior colliculus that are observed in this particular disorder.

1. Introduction

Oxidative stress resulting from impaired cerebral mitochondrial function is a hallmark of some acute as well as chronic neurodegenerative disorders such as ischemia, stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (review in Fiskum et al., 1999) and Wernicke's encephalopathy (Butterworth et al., 1993; Leong and Butterworth, 1996). Interestingly, most of these neurodegenerative disorders show several common dysfunction including inhibition of the α-ketoglutarate dehydrogenase complex (αKGDH), which is a rate limiting enzyme in the citric acid cycle and whose function is severely compromised by several factors (review in Gibson et al., 2000). Wernicke's encephalopathy is a unique neurodegenerative disorder caused by a nutritional/cofactor, namely thiamine or vitamin B1 deficit. It leads to impaired mitochondrial oxidative metabolism and consequent neuronal death (Heroux and Butterworth, 1992), which is often reversed by the timely administration of the thiamine cofactor (Sparacia et al., 1999).

In humans, this disease leads to a syndrome often characterized by ophthalmoplegia, ataxia, and memory loss. Neuropathological evaluation shows extensive neuronal cell loss and panecrosis in the thalamic nuclei, mammillary bodies, and cerebellum with relative sparing of cerebral cortical structures (Harper and Butterworth, 1997).

Pyrithiamine induced thiamine deficiency (PTD) in the rat is a well validated experimental model to study the pathophysiology of human Wernicke's encephalopathy by

the nature of its biochemical and region selective histological lesions which mirror those seen in the human disorder (Troncoso et al., 1981). The earliest metabolic dysfunction manifested in PTD is the inhibition of αKGDH (Butterworth et al., 1986; Butterworth and Heroux, 1989), an event which results in altered mitochondrial function and is further responsible for the propagation of a cascade of abnormalities leading to oxidative stress.

Numerous mechanisms have been proposed to explain the selective neuronal loss found in TD. They include cerebral energy dysfunction (Aikawa et al., 1984), breakdown of the blood-brain barrier (Calingasan et al., 1995a; Harata and Iwasaki, 1995), altered glutamate neurotransmission caused by increased extracellular glutamate and its impaired transporter function (Hazell et al., 1993, 2001), NMDA receptor-mediated excitotoxicity (Langlais and Mair, 1990), accumulation of amyloid precursor like protein (Calingasan et al., 1995b), increased free radical production (Langlais et al., 1997), increased expression of superoxide dismutase (Todd and Butterworth, 1997), consequent to increased microglial response (Todd and Butterworth, 1999), induction of nitric oxide (Calingasan et al., 1998), and oxidative stress (Langlais et al., 1997; Desjardins and Butterworth, 2003).

As evidence for the increase in oxidative stress, increased nitrotyrosine immunolabelling of neurons in the classically vulnerable brain regions in the thiamine deficiency model have been observed (Calingasan et al., 1998). On the other hand, when total nitric oxide synthase (NOS) activities have been measured enzymatically (Rao et al., 1996), or the densities of NOS-containing neurons have been identified by NADPH-

diaphorase staining (Matsushita et al., 2000), they are significantly downregulated in the targeted brain regions in the experimental model of TD.

Three distinct isoforms of NOS have been recognized; an inducible NOS (iNOS), which is usually found in microglia and astrocytes in the brain; a constitutive neuronal isoform (nNOS); and the endothelial isoform (eNOS). The purpose of the present study was to determine the time-course of alterations in expression of the eNOS isoform in brain structures shown previously to be selectively vulnerable to TD. For this purpose we have studied the differences in mRNA and protein expression of eNOS in the characteristically vulnerable regions of the rat brain, using the pyrithiamine-induced thiamine deficiency model in the rat.

2. Experimental procedures

2.1. Animal surgery

Adult male Sprague Dawley rats weighing 200-225 g obtained from Charles River (St. Constant, Quebec, Canada) were used in all experiments. Rats were housed individually under constant conditions of temperature, humidity and 12 hours light/dark cycles and had free access to water at all times. Rats were allowed to adapt to their environment for 3 days prior to the initiation of treatments. At the initiation of the experiments, animals were assigned to either thiamine-deficient or pair-fed control groups. All animal treatment procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital and the University of Montreal.

2.2. Thiamine deficiency protocol

Rats in the thiamine deficient group (TD) were fed a thiamine-deficient diet (ICN Nutritional biochemicals, Cleveland, OH, USA) and administered daily pyrithiamine hydrobromide (0.5 mg per kg body wt) intraperitoneally. Control rats were pair-fed to equal food consumption with the TD rats using the same thiamine-deficient food and were supplemented with daily i.p. injections of thiamine (0.1 mg per kg body wt). After 12 days of treatment, rats were randomly assessed for neurological abnormalities and selected to establish a pre-symptomatic stage by careful observation of characteristic symptoms, such as development of rotating movement and/or tendency towards backward movement without any seizures or loss of righting reflex (Hazell et al, 1998). Treatments were continued in the rest of the groups until rats displayed specific behaviors such as loss of righting reflex (where the animal is no longer able to right itself when placed on its back). This stage was considered as the acute symptomatic stage. Any rats exhibiting spontaneous seizures were eliminated from the protocols. The animals were sacrificed by decapitation. The brains were promptly removed and flash frozen in isopentane on dry ice and stored at –80 ^{o}C. They were then dissected on ice into three different regions (i.e. frontal cortex, medial thalamus and inferior colliculus) according to the rat brain atlas of Paxinos and Watson (1986).

2.3. Protein extraction

Medial thalamus, frontal cortex, and inferior colliculus tissue samples were homogenized at 4°C in 50 mM Tris-HCl buffer pH 7.4, containing a protease inhibitor cocktail (Sigma, St.Louis, MO, USA) using a Potter-Elvehjem tissue homogenizer. After centrifugation of samples at 12,000g for 45 minutes, membranes were separated from cytosolic fraction, washed in the same buffer and stored at –80°C. Protein concentrations were estimated based on the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.4. Western blot analysis

Membrane fractions from the medial thalamus, frontal cortex, and inferior colliculus (10-100 μg protein equivalent) were solubilized in Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1M dithiothreitol, 0.1% bromophenol blue) and boiled for 10 minutes. Proteins were resolved on 7.5% denaturing SDS-polyacrylamide gels and transferred overnight at 4°C to PVDF membranes. Membranes were blocked by incubation for two hours at room temperature in Tris buffered saline containing 0.05% Tween 20 (TBST) and 5% dry milk then incubated for 2 hours with a monoclonal antibody directed against eNOS (1:1000 dilution; Pharmingen, San Diego, CA, USA) or β-actin (1:75,000, Sigma). Membranes were washed several times with TBST and incubated for 1 hour with an anti-mouse-horseradish peroxidase (HRP)-

conjugated secondary antibody (PerkinElmer Life Sciences, Boston, MA, USA). After extensive washing with TBST, peroxidase activity was detected by enhanced chemiluminescence using ECL detection system (Amersham, Arlington Heights, IL, USA).

2.5. RNA extraction

Total RNA was extracted from the medial thalamus, frontal cortex, and inferior colliculus using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Putative contaminating DNA was eliminated by adding 100 U of RNase-free DNase I per 50 μg of total RNA at 37°C for 1 h. Purified RNA was then extracted with phenol, precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water. RNA samples were stored at -70°C until use.

2.6. RT-PCR analysis

Expression of eNOS was investigated by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA (1 μg) was mixed with 10 mM Tris-HCl, pH 8.3 buffer containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) BSA, 100 μM dNTPs, primers at 1 μM each, AMV reverse transcriptase (80U/ml), Taq DNA polymerase (20 U/ml) and 50 μCi/ml [α-32P]dCTP (3000 Ci/mmol), in a total reaction volume of 50μl. Samples were initially heated at 50°C for 15 min followed by PCR at 95°C for 30 sec, 60°C for 45 sec and 72°C for 1 min. Amplification efficiency conditions were determined after a kinetic study to ensure all experiments were performed within the exponential phase of amplification, where PCR product remains proportional to initial template concentration (data not shown).

In all the experiments, β-actin was an internal standard to monitor loading variations. β-actin and eNOS, were amplified for 21 and 28 cycles respectively. After amplification, samples were electrophoresed onto 8% polyacrylamide gels, dried and autoradiographed at –80°C with intensifying screens. Each corresponding band was excised and Cerenkov radiation was quantified using a β-counter. Oligonucleotide primers were designed using the PRIME program (Genetic Computer Group, Wisconsin) and synthesized by the Sheldon Biotechnology Center (McGill University, Montreal, Canada) based on the following gene sequence from GeneBank accession numbers: V01217 (β-actin, Nudel et al., 1983) with forward and reverse primer sequence 5'-CATCCCCCAAAGTTCTAC-3' and 5'-CCAAAGCCTTCATACATC-3' (347 bp), and U53142 (eNOS, Gnanapandithen et al., 1996), 5'-TCAGTGGCTGGTACATGAG-3' and 5'-ACAGGAAATAGTTGACCATC TC-3' (351 bp). The specificity of oligonucleotide primers was verified using BLASTN (National Center for Biotechnology Information, Bethesda, MD).

2.7. Immunohistochemisty

Rats whose brains were processed for immunohistochemical evaluation were anaesthetized with pentobarbital (80mg/kg) and perfused transcardially with 150 ml of saline followed by 150 ml of neutral-buffered formalin containing 4% formaldehyde, 0.5% sodium phosphate buffer, 1.5% methanol and 0.02% glutaraldehyde, pH 7.0 (Fisher Scientific, Fair Lawn NJ). The brains were then removed and post-fixed overnight in the same solution. Paraffin-embedded coronal sections of 6μm thickness were cut at the level

of the frontal cortex and medial thalamus, and mounted on slides coated with Vectabond Reagent (Vector Laboratories, Burlingame, CA). Slides were deparaffinized by heating at 60°C for 30 minutes followed by xylene, and rehydrated using consecutive baths of ethanol 100%, 95%, 85%, 70%, and distilled water. Endogenous peroxidase was blocked by means of 1% H_2O_2 in methanol for 30 minutes at room temperature and then washed with PBS. For eNOS antigen retrieval (AR), the slides were transferred into microwaveable-glass baths containing 5% urea/Tris-HCl buffer, pH 9.5, as formerly described by Shi et al., (1996). The baths were heated in a microwave oven twice for 5 minutes (Sanyo Model EM-802TW, operating at 2450 MHz with the highest power setting 600W). When necessary, more AR buffer was added after the first 5 minutes to compensate for loss due to boiling over and to avoid drying tissue sections. After heating, slides remained in the baths for an additional 30 minutes before washing in distilled water, followed by PBS. The non-specific binding sites were blocked using 10% normal horse serum in 0.5% Triton X-100/PBS for 30 minutes. The slides were incubated overnight with eNOS monoclonal antibody (1:250, Pharmingen) in a humid chamber at 4°C. After washing in PBS, the sections were then incubated with biotinylated horse anti-mouse IgGs secondary antibody (1:100, Vector Laboratories), followed by incubation with ABC reagent (Vector Laboratories). eNOS immunoreactivity was subsequently detected by incubation with 3-3'-diaminobenzidine (DAB) containing urea hydrogen peroxide (Sigma). The slides were counterstained with cresyl violet and mounted for examination. Negative control slides were treated identically except that the primary antibodies were omitted.

2.8. Statistical analysis

Statistical analysis was performed using by two-way analysis of variance (ANOVA) with Newman Keuls post hoc analysis to establish significance between comparisons. A probability of $p<0.05$ was chosen to establish significance between the groups.

3. Results

After 11 days of treatment, pyrithiamine-treated animals exhibited changes in behavior that consisted of rotational and/or backward movements without displaying any signs of ataxia or opisthotonic episodes, which were consistent with earlier studies (Hazell et al., 1998). This was followed on days 13 and 14 by severe ataxia or opisthotonic episodes lasting for 18-24 hours, followed by complete loss of righting reflex. Time course study showed no macroscopic brain lesions prior to day 12. After day 12, petechial lesions were observable in the medial thalamus and inferior colliculus on which the present study focuses. Edema was also noticeable at day 12. The consistency of the region-specific lesions allows for study of neuropathological changes throughout the chosen time course.

Immunohistochemical staining was performed to investigate eNOS expression in the frontal cortex and medial thalamus of symptomatic TD rats. Endothelial cells of the medial thalamus from symptomatic rats showed increase eNOS immunoreactivity compared with basal (constitutive) level of expression in the pair-fed group (data not shown). eNOS immunoreactivity in the medial thalamus of symptomatic rats was more

intense and also localized to larger blood vessels when compared to the frontal cortex, a spared region in thiamine deficiency (Figure 1).

Figure 1

Western blot analysis of eNOS (Figure 2) revealed a minor increase in the pre-symptomatic stage when compared to the pair-fed control group and showed a statistically significant increase in the acute symptomatic stage (2-3 fold, $p<0.01$). There was no significant variation of eNOS in the non-vulnerable frontal cortex region.

Figure 2

RT-PCR analysis of eNOS mRNA expression in the inferior colliculus and medial thalamus revealed, after normalization to β-Actin mRNA, the same trend as for eNOS protein expression, i.e. being significantly increased in the medial thalamus (3 fold, $p<0.001$) and inferior colliculus (2 fold, $p<0.05$) in the fully symptomatic animals. As expected, eNOS protein expression in the frontal cortex showed no significant alterations.

Figure 3

4. Discussion

We found that mRNA and protein expression of the eNOS isoform were increased in a selective manner in two of the characteristically vulnerable regions in this pathology, i.e. showing severe neuronal cell loss (Heroux and Butterworth, 1992), namely the medial thalamus and inferior colliculus. This increased expression was apparent as of day 12 of pyrithiamine-induced thiamine deficiency treatment, and these effects took place prior to the onset of severe neurological symptoms and also of the usual thalamic lesions found in this particular animal model (Todd and Butterworth, 1998; Calingasan et al., 1998).

As the pathology advanced, the resulting thiamine deficiency led to ataxia, loss of righting reflex, and a further increase in eNOS expression in the inferior colliculus and medial thalamus, two of the typically affected neuroanatomical structures. There was no significant increase observable in the expression of eNOS in the commonly non-vulnerable frontal cortex region.

Interestingly, in contrast to this increase in expression of the endothelial isoform, the expression of the other two isoforms (iNOS and nNOS) presented no significant changes in either the medial thalamus or the inferior colliculus of the pre-symptomatic or acute symptomatic PTD- treated animals when the protein or mRNA expression were measured by Western blot or RT-PCR respectively (data not shown).

These findings indicate that if NO is implicated in the pathogenesis of neuronal cell death caused by thiamine deficiency, it is likely a product of its increased output from vascular endothelial cells. Indeed, there is evidence from previous studies that has indicated

that the vascular endothelium is a major site of free radical production in areas of neuronal cell loss in both experimental TD and Wernicke Encephalopathy in humans (Calingasan and Gibson, 2000; Okeda et al.,1995).

Studies made in a related pathology, experimental cerebral ischemia, tend towards the conclusion that eNOS might have a neuroprotective function, which is an effect that might be caused by its role in modifying presynaptic signals, and this leads to an increase in GABA release (Kano et al.,1998). Other studies made in cultured cells indicate that NO derived from eNOS limits apoptotic cell death (Estevez et al.,1998). The process of apoptotic cell death has been observed in the thalamic region in experimental TD (Matsushima et al.,1997), but the extent to which apoptotic cell death is modulated by the increase in expression of eNOS in thiamine deficiency has not yet been explored. On the other hand, it has been demonstrated that a targeted disruption of the eNOS gene leads to a reduction in the severity of the neuronal damage in a mouse model of TD (Calingasan et al., 2000), which points towards the theory that eNOS-derived NO is in fact neurotoxic rather than neuroprotective.

The increased production of NO by eNOS is a plausible explanation for previous findings of an increase in nitrotyrosine immunolabelling in experimental TD (Calingasan et al., 1998), and the increase in NO production could also relate to a recent report of selective decreases in the expression of cytochrome C oxidase (CCO) in the medial thalamus of TD animals (Rama Rao et al., 2000). Increased eNOS expression observed in the present study showed comparable regional and temporal profiles to those reported for CCO. These

findings might be of pathophysiological importance since an inhibitory effect of NO on CCO expression has been observed in isolated mitochondria and in various neuronal preparations (Brown, 2001). The decrease in the activity of CCO activity has been suggested as a possible cause of neuronal cell death in several neurodegenerative disorders (Kish et al., 1992) and the results obtained in the present study indicate that eNOS-derived NO production, through the production of toxic intermediates such as peroxynitrites, or by inhibition of CCO and mitochondrial processes, could be a factor in the pathogenesis of the selective neuroanatomical lesions seen in syndromes involving TD including Wernicke encephalopathy in humans.

Prior studies in TD have reported enhanced immunoreactivity to NOS in a variety of cell types (Calingasan et al., 1998, 1999). The results obtained in the present study, show an increase in eNOS mRNA, which precedes an increase in eNOS protein expression in the acute symptomatic stage. This extends earlier immunohistochemical studies (Calingasan et al., 1998) and demonstrates early, region-selective, increases of eNOS gene expression in TD.

The finding is important since it was previously proposed that NO derived from iNOS rather than eNOS is a source of the ensuing neurotoxicity in several neurodegenerative diseases such as Alzheimer's disease (Vodovotz et al., 1996) as well as in cerebral ischemia (Endoh et al., 1994). Increased iNOS expression is plausible in these particular disorders because under pathological conditions (e.g. injury or metabolic insult), reactive gliosis could lead to an inflammatory-type of reaction causing induction of iNOS.

On the other hand, Calingasan et al. (1999) observed that iNOS knockout mice were not protected from the deleterious effects of TD such as extensive neuronal loss and enhanced production of free radical species. Rather, the targeted deletion of the eNOS gene reduced the neurodegeneration and oxidative stress in TD mice. These findings are all supportive of the notion that selective eNOS rather than iNOS induction is a key factor in TD leading to oxidative stress and neuronal cell damage and loss.

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Legends to Figures:

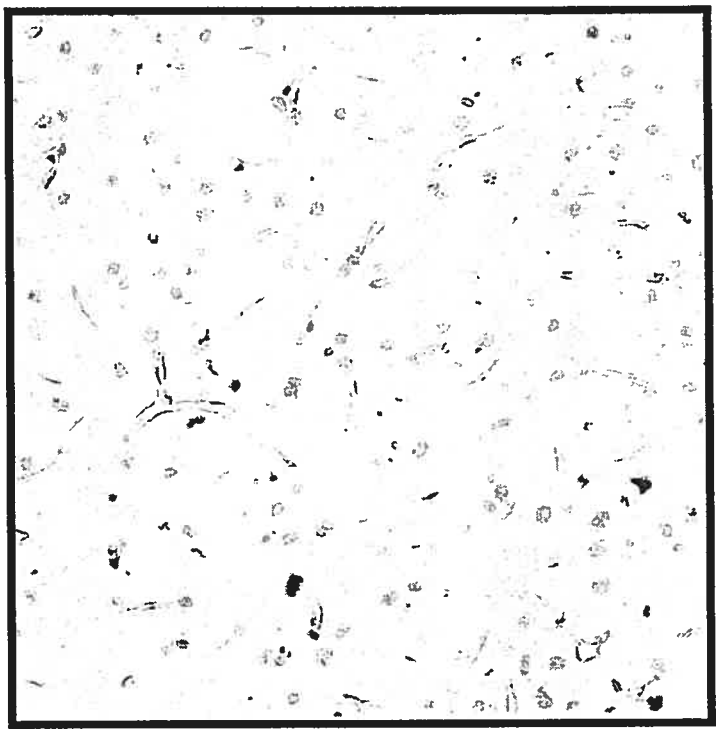
Figure 1. Endothelial nitric oxide (eNOS) immunolabeling in the frontal cortex (A) and medial thalamus (B) of thiamine deficient rats at symptomatic stage. Paraffin-embedded sections were probed with eNOS monoclonal antibody. Immunoreactivity was detected using horseradish peroxidase-coupled anti-mouse IgGs and diaminobenzidine. Sections were counterstained with cresyl violet. Control sections were incubated with normal sera instead of primary antibody; these showed absence of immunostaining.

Figure 2. Western blot analysis of endothelial nitric oxide synthase (eNOS) in the medial thalamus (A), inferior colliculus (B), and frontal cortex (C) of thiamine deficient rats. Membrane fraction was prepared from dissected brain regions of thiamine-deficient rats at presymptomatic (12 days of treatment) and symptomatic stages (loss of righting reflex) or from pair-fed controls. Proteins were resolved on 8% SDS-polyacrylamide gels, transferred to PVDF membranes and probed with a monoclonal antibody against eNOS. Pair-fed controls (PFC); presymptomatic rats (PS); symptomatic rats (SYM). Results shown are representative of duplicate experiments performed with four rats in each group.

Figure 3. Expression of endothelial nitric oxide synthase (eNOS) mRNA in the medial thalamus (A), inferior colliculus (B), and frontal cortex (C) of thiamine deficient rats. Total RNA was extracted from dissected brain regions of thiamine-deficient rats at presymptomatic (12 days of treatment) and symptomatic stages (loss of righting reflex) or

from pair-fed controls. ß-Actin (347 bp) and eNOS (351 bp) were reverse-transcribed and amplified by PCR for 21 and 28 cycles respectively. Lane 1: molecular weight standard; lanes 3-6: pair-fed controls; lanes 7-10 presymptomatic rats; lanes 10-14: symptomatic rats; lane 2: reverse transcriptase was omitted from the reaction mixture as negative control.

(A)



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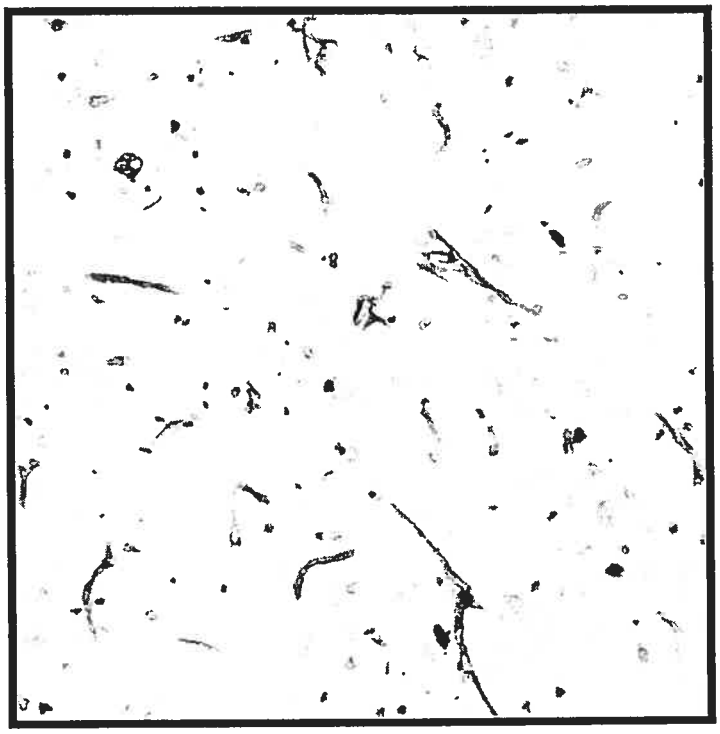
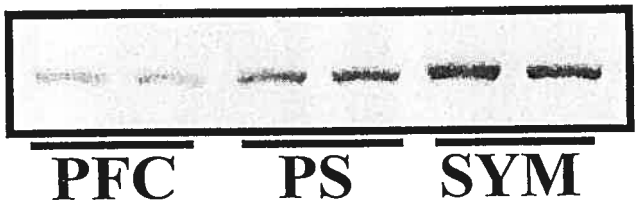
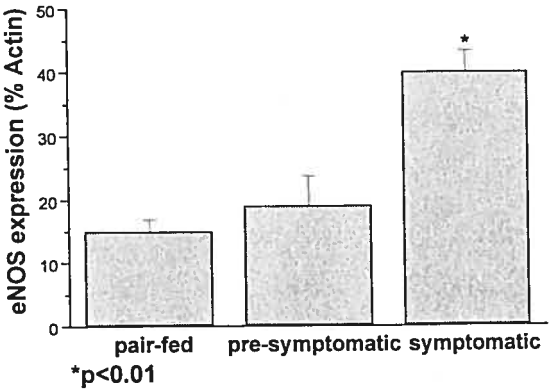


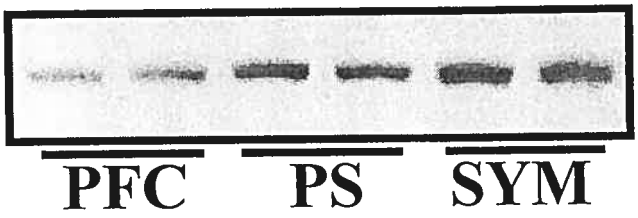
(A)

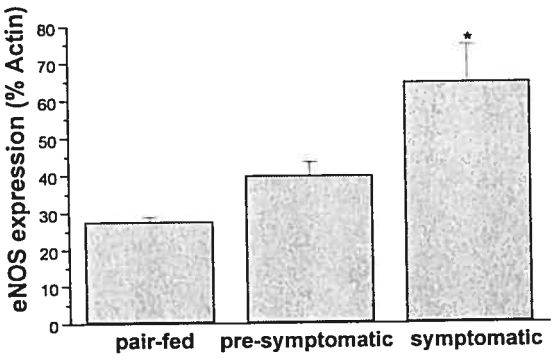




*p<0.01

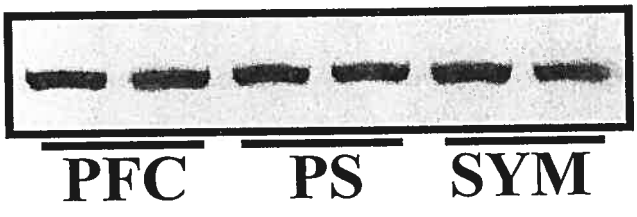
(B)

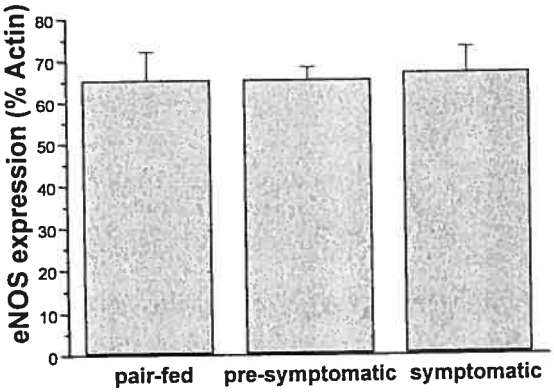


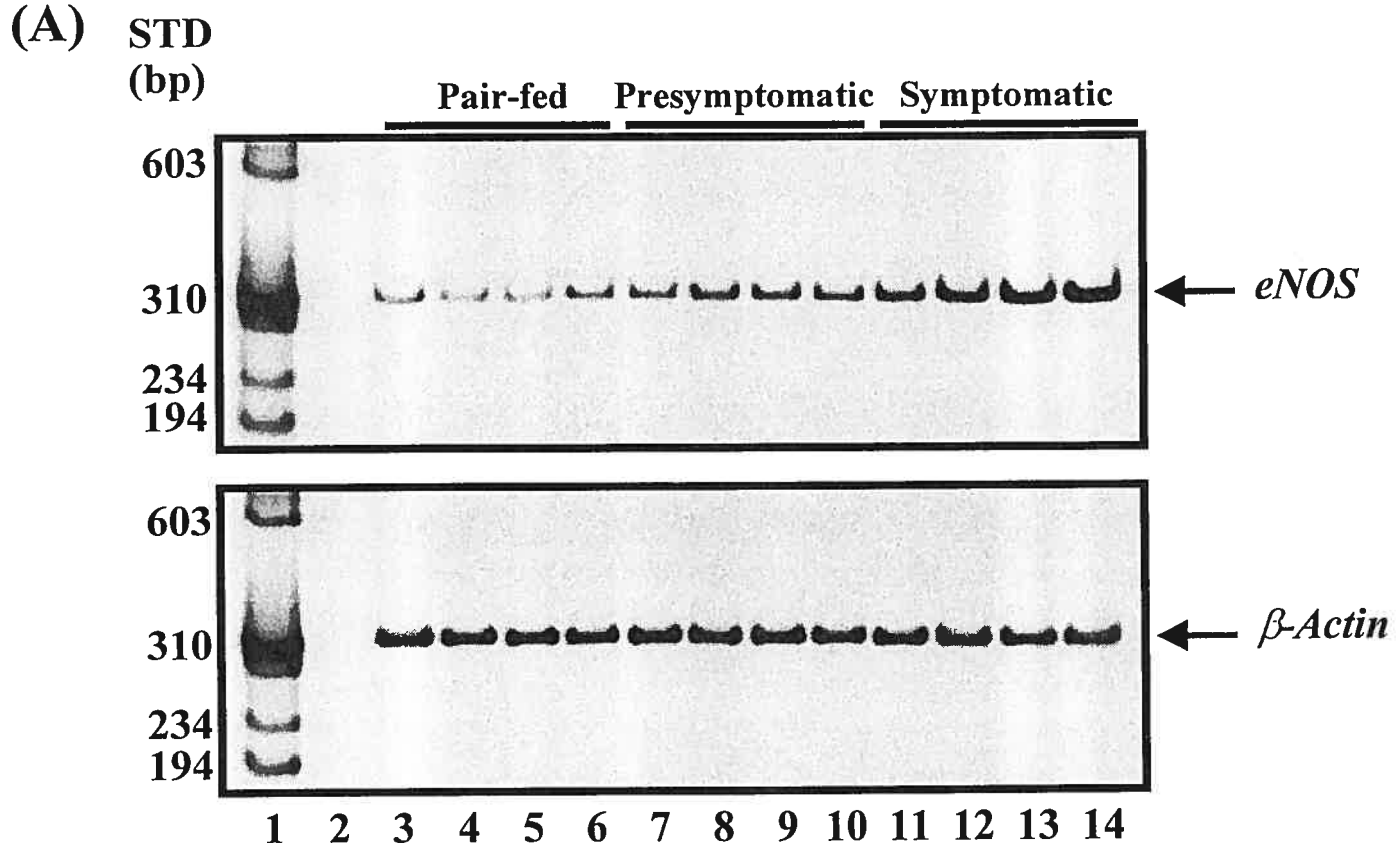


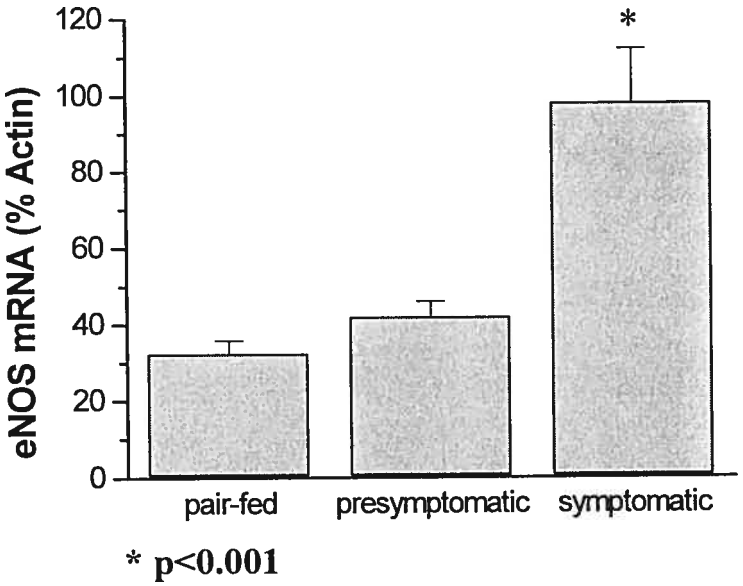
*p<0.01

(C)



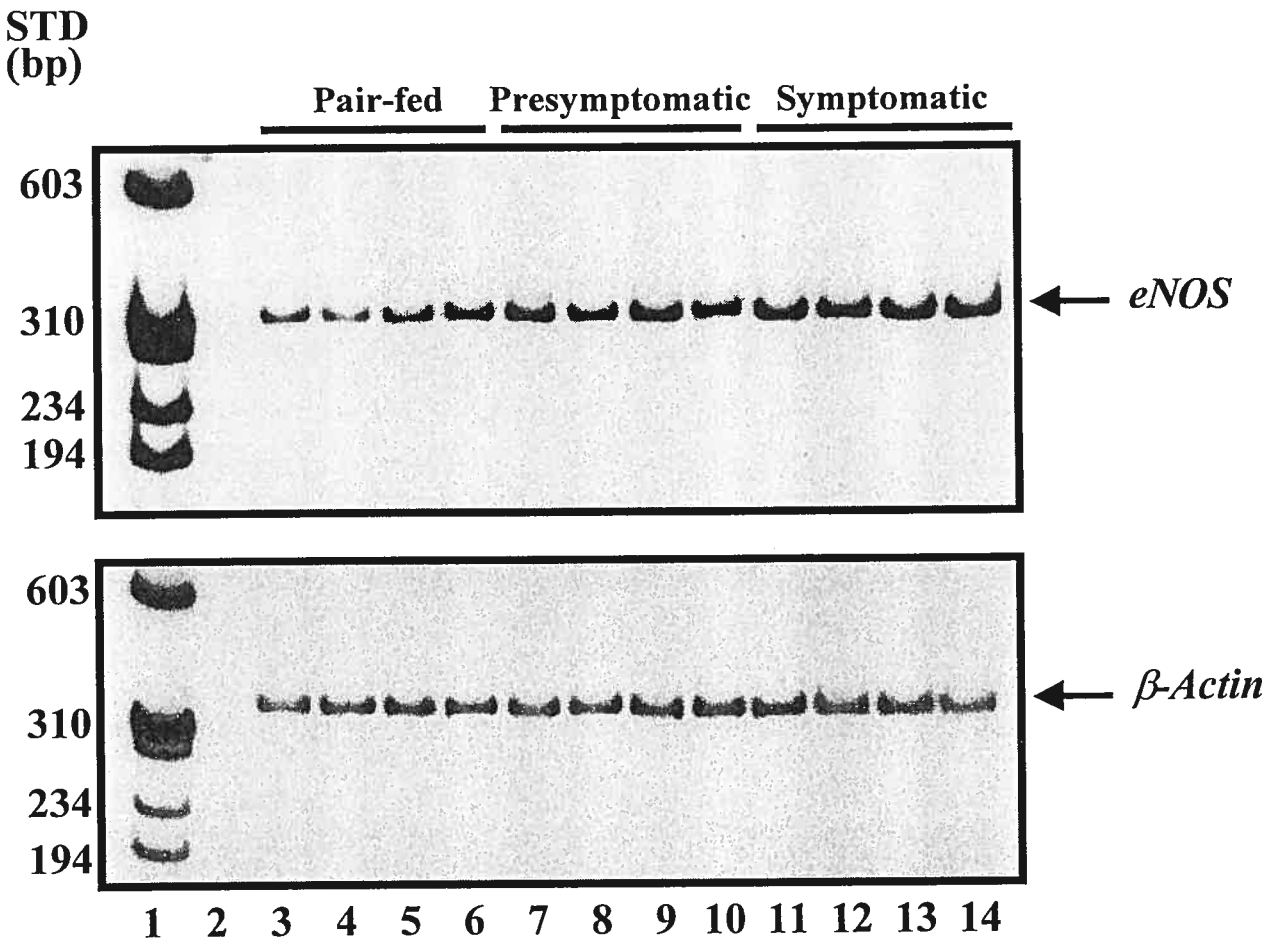



(A)
STD
(bp)
Pair-fed
Presymptomatic
Symptomatic
603
310
234
194
eNOS
603
310
234
194
β-Actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14


eNOS mRNA (% Actin)
120
100
80
60
40
20
0
*
pair-fed
presymptomatic
symptomatic

* p<0.001

(B)


STD
(bp)
Pair-fed
Presymptomatic
Symptomatic
603
310
234
194
eNOS
603
310
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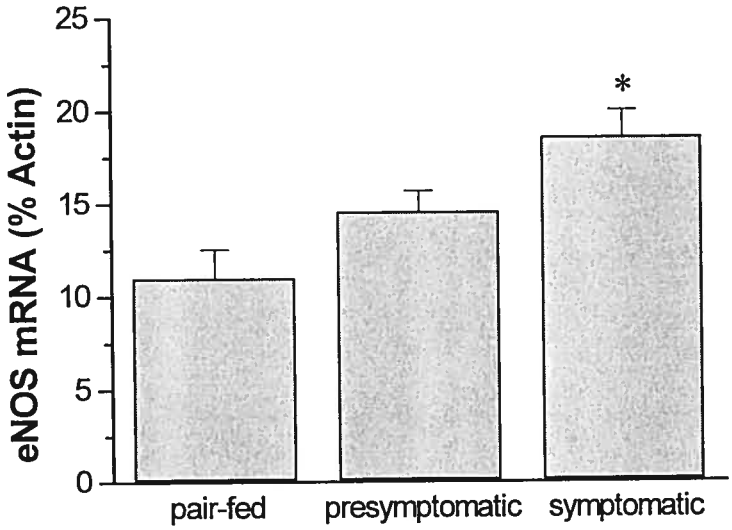
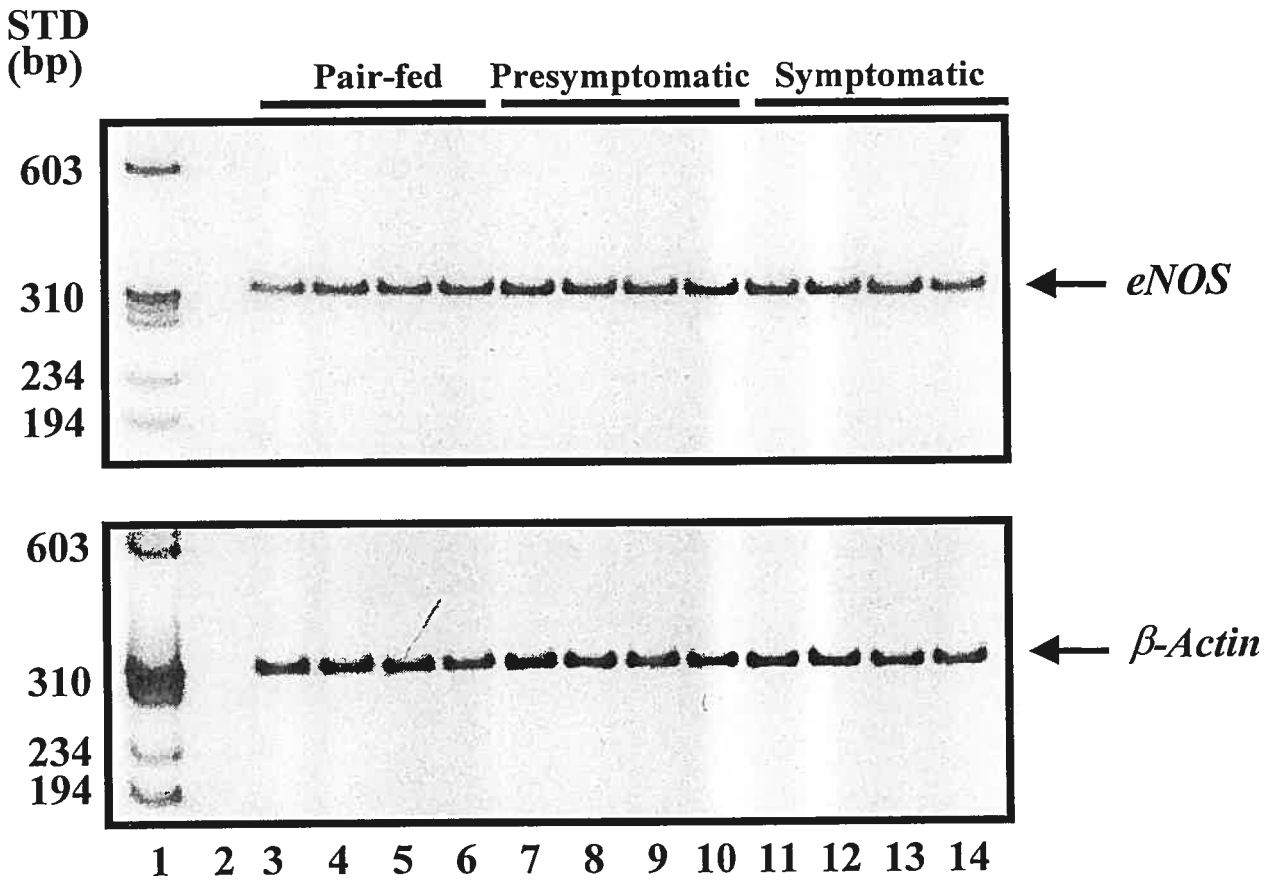
β-Actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14


eNOS mRNA (% Actin)
25
20
15
10
5
0
*
pair-fed
presymptomatic
symptomatic

*** p<0.05**

(C)


STD
(bp)
Pair-fed
Presymptomatic
Symptomatic
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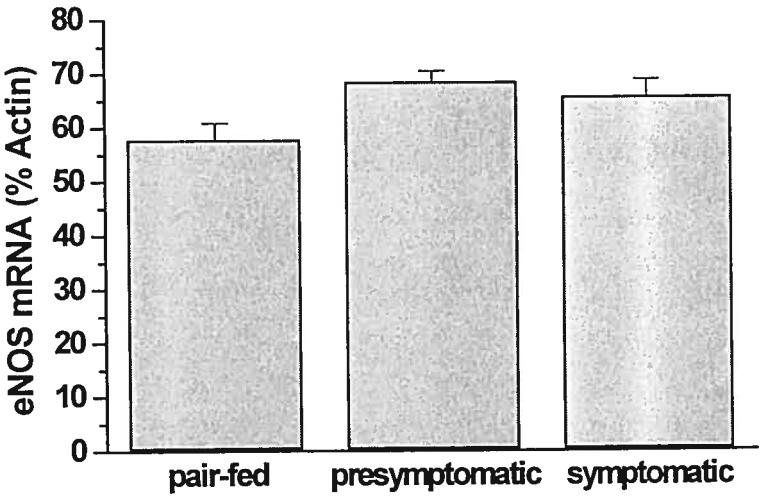
β-Actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14


eNOS mRNA (% Actin)
80
70
60
50
40
30
20
10
0
pair-fed
presymptomatic
symptomatic

Conclusions

3.1 Conclusions

Les expressions de l'ARN messager et de la protéine du NOS endothélial ont été sélectivement aumentées dans les deux régions caractéristiquement vulnérables dans la DTP, c'est-a-dire, le thalamus médian et le collicullus inférieur. Les expressions de L'ARN messager et de la protéine dans le eNOS sont restées sans changement dans le cortex frontal.Cette augmentation des expressions a été observée depuis le jour 12 du traitement de déficience en thiamine induite par la pyrithiamine, et ces changes ont apparut avant la manifestation des symptomes neurologiques graves (Todd et Butterworth, 1998; Calingasan et al., 1998).

Le processus pathologique induit par le traitement a continué et avec l'avancement de la déficience en thiamine les animaux ont démontré des symptomes classiques tels que l'ataxie, la perte du réflexe de redressement et une augmentation dans l'expression du eNOS dans le colliculus inférieur et dans le thalamus médian visible au stage pré-symptomatique et augmentant de façon significative lors du stage symptomatique. L'expression dans la protéine des autres isoformes du monoxyde d'azote (iNOS et nNOS) resta sans changements consistants quand elle a été observée par moyen du Western Blot.

Nos résultas semblent indiquer que si le monoxyde d'azote est impliqué dans le proces de la mort cellulaire neuronale causée par la déficience en thiamine, ceci est le produit de l'augmentation du NO provenant des cellules vasculaires endothéliales.

Effectivement, il existe plusieurs études qui démontrent que l'endothélium vasculaire est un site important de production de radicaux libres dans les régions de

perte neuronale dans la déficience en thiamine et aussi dans l'EW chez l'humain. (Calingasan et Gibson, 2000; Okeda et al., 1995).

Dans une étude récente, il a été démontré qua l'intérromption spécifique du gene de eNOS limite les dommages neuronaux dans le modele de la souris utilisé dans la DT (Calingasan et al., 2000), ce qui indique que le monoxyde d'azote ayant pour source le eNOS est plutôt neurotoxique et pas neuroprotecteur, ce qui est le cas dans d'autres pathologies neurodégénératives telles que l'ischimie.

Ces trouvailles sont importantes car il est généralement pensé que le NO ayant pour source le iNOS et pas le eNOS est la cause de la neurotoxicité retrouvée dans plusieurs maladies neurodégénératives telles que la maladie d'Alzheimer (Vodovotz et al., 1994) et l'ischimie (Endoh et al., 1994). Dans ce maladies, ceci prends place car dans des conditions pathologiques (ex. dommages ou insulte métabolique), il y aurait une prolifération de gliose réactive, ce qui entraîne une réaction de type inflammatoire qui causerait l'induction du NO par l'iNOS.

D'après nos résultas et l'evidence montrée par les études mentionnées, ont peut suggèrer que l'inhibition spécifique du NOS endothélial serait un traitement potentiel a pousuivre pour réduire la perte neuronale dans les cas avancés d'encéphalopathie de Wernicke.

3.2 Conclusion

mRNA and protein expression of the eNOS isoform were selectively increased in the vulnerable brain regions in PTD, namely the medial thalamus and the inferior colliculus. The mRNA and protein expression of eNOS remained unchanged in the frontal cortex. This increased expression of eNOS was observed starting on day 12 of pyrithiamine-induced thiamine deficiency, before the onset of severe neurological symptoms. As the thiamine deficiency developed, the animals

displayed ataxia, loss of righting reflex, and an increase in the expression of eNOS in the inferior colliculus and medial thalamus that started at the presymptomatic stage and significantly increased with the onset of the acute symptomatic stage.

The protein expression of the other isoforms of NOS (nNOS and iNOS) did not display any significant or consistent changes in any region at either the presymptomatic or acute symptomatic stages when assayed by Western Blotting. These findings indicate that if NO is implicated in the pathogenesis of neuronal cell death caused by thiamine deficiency, it is most likely a product of its increased output form vascular endothelial cells.Indeed, a growing body of evidence from previous studies suggests that the vascular endothelium is a major site of oxidative free radical production in areas of neuronal cell loss in both thiamine deficiency and WE in humans (Calingasan and Gibson, 2000; Okeda et al., 1995).

Studies undertaken in a related pathology, namely experimental cerebral ischemia, indicate that eNOS may serve a neuroprotective function, which could be an effect related to its role in modifying presynaptic signal, leading to an increase in GABA realease (Kano et al., 1998). Studies made in cultured cells indicate that eNOS-derived NO limits apoptotic cell death (Estevez et al., 1998). The process of apoptotic cell death has been observed in the thalamic region in experimental thiamine deficiency (Matsushima et al., 1997), but the extent to which apoptotic cell death is modulated by the increase in expression of eNOS in thiamine deficiency requires further investigation.

It was previously shown that the specific disruption of the eNOS gene limits the extent of the neuronal damage in the murine model of TD (Calingasan et al., 2000), which indicates that eNOS-derived NO is rather neurotoxic than neuroprotective in this experimental situation. The rise in production of NO following eNOS induction could explain the previous findings of an increase in nitrotyrosine immunolabelling in experimental TD (Calingasan et al., 1998), and the increase in NO production

could also explain a recent finding that reported selective decreases in the expression of cytochrome C oxidase (CCO) in the medial thalamus of TD animals (Rama Rao et al., 2002). In support of this, the increased eNOS expression observed in the present study showed comparable regional and temporal profiles to those reported in the CCO studies. These findings could be of pathophysiological importance since an inhibitory effect of NO on CCO expression has been observed in isolated mitochondrion and various neuronal preparations (Brown, 2001). The decrease in the activity of CCO activity has been suggested as a possible cause of neuronal cell death in several of the neurodegenerative disorders (Kish et al., 1992) and the results obtained in the present study suggest that eNOS-derived NO production might, through the production of deleterious intermediates such as peroxynitrites, or by inhibition of CCO and mitochondrial processes, lead to the development of the selective lesions seen in thiamine deficiency and, by inference, in Wernicke encephalopathy in humans.

The earliest pathological event described in this model is the breakdown of the blood-brain barrier (BBB), which could produce increased NO via eNOS. Several recent studies have tried to clarify which of the two isoforms might play a more significant role in the development of the lesions. Calingasan et al. (1999) demonstrated that in the case of iNOS knockout transgenic mice, these animals were not protected from the deleterious affects of TD treatment such as extensive neuronal loss and enhanced production of free radical species. In this same study, the specific deletion of the eNOS gene limited both the neurodegeneration and oxidative stress in TD mice. These findings suggest that NO derived from eNOS and not iNOS leading to oxidative stress damage, could contribute to the development of specific lesions in TD pathologies.

The results obtained support the hypothesis that a systemic metabolic injury caused by generalized thiamine deficiency increases NO production and a variety of other free radical producers in the endothelial cells of the BBB, which leads to the

malfunction of this neuroprotective mechanism. The breakdown in the BBB would then allow the entry of large and deleterious molecules such as iron into the brain. The entry of these molecules in conjunction with the metabolic alterations initiates the activation of microglia, which consequently begin an inflammatory process. Indeed, previous studies (Calingasan et al. 2000) have also demonstrated that vascular factors are critical in the pathological sequence that terminates in oxidative stress and death of metabolically impaired neurons in the histologically vulnerable regions in TD. TD induced ICAM-1 and in the late stages of the pathology, eNOS are induced in the microvessels of the thalamic region. The use in these studies of mice devoid of the ICAM-1 and/or eNOS genes led to significant blunting of the neuropathology in this region.

These findings provide further evidence that endothelial cells are an important site for inflammatory processes and that they are also significant sources of free radical production in the sensitive regions of the brain of the PTD animal. Pharmacological inhibition studies of eNOS would serve to better elucidate the role of this isozyme in the PTD pathology cascade.

In summary, an increase in eNOS production in the vulnerable regions of the brain might be a contributing factor to neuronal cell death by means of increased production of NO and other free radical species and thus to oxidative stress processes.

This increase is likely to be the product of the loss of integrity of the blood-brain barrier in TD and its associated pathologies.

Since these findings indicate that an increase in eNOS production might be indeed more deleterious than protective in some neurodegenerative diseases, selective inhibition of this isoform should be pursued as a potential treatment in advanced cases of these diseases in the future since it might confer neuronal protection.

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