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Update of Cell Damage Mechanisms in Thiamine Deficiency: Focus on Oxidative Stress, Excitotoxicity and Inflammation

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Abstract — Thiamine deficiency (TD) is a well-established model of Wernicke's encephalopathy. Although the neurologic dysfunction and brain damage resulting from the biochemical consequences of TD is well characterized, the mechanism(s) that lead to the selective histological lesions characteristic of this disorder remain a mystery. Over the course of many years, various structural and functional changes have been identified that could lead to cell death in this disorder. However, despite a concerted effort to explain the consequences of TD in terms of these changes, our understanding of the pathophysiology of this disorder remains unclear. This review will focus on three of these processes, i.e. oxidative stress, glutamate-mediated excitotoxicity and inflammation and their role in selective vulnerability in TD. Since TD inhibits oxidative metabolism, a feature of many neurodegenerative disease states, it represents a model system with which to explore pathological mechanisms inherent in such maladies, with the potential to yield new insights into their possible treatment and prevention.

INTRODUCTION

In 1881, Carl Wernicke provided the first report of a disorder in three patients characterized by ataxia, ophthalmoplegia and mental changes, an illness that would later bear his name, that of Wernicke's encephalopathy (WE) (Wernicke, 1881). One of two components of the Wernicke–Korsakoff syndrome (WKS), a neuropsychiatric disorder characterized by ophthalmoplegia, gait ataxia and confusion/memory loss, WE is caused by thiamine deficiency (TD). Analysis of available data suggest that WE occurs in chronic alcoholics at a frequency of ∼35%, and in the population as a whole, the figure is ∼1.5% (Cook *et al*., 1998). Current evidence also suggest that TD occurs in chronic alcoholics at a frequency of at least 25–31% (Baines, 1978; Camilo *et al*., 1981; Lévy *et al*., 2002) and up to 80% (Thomson *et al*., 1987; Morgan, 1999). WE is therefore a common complication of chronic alcoholism, and other disorders associated with grossly impaired nutritional status that include gastrointestinal disorders (Lindboe and Loberg, 1989), hyperemesis gravidarum (Ebels, 1978; Ohkoshi *et al*., 1994), HIV AIDS (Soffer *et al*., 1989; Butterworth *et al*., 1991) and malignant disease (Shah and Wolff, 1973; Miyajima *et al*., 1993).

Focal cerebral vulnerability is a major consequence of TD. Neuropathologic evaluation of brain tissue from WE patients reveals a clear pattern of selective damage to subcortical areas of the brain that includes the thalamus and mammillary bodies, along with the midbrain inferior colliculus, and brainstem structures that include the vestibular nuclei and olivary complex (Harper and Butterworth, 1997). Previous studies have established decreased activities of the thiamine-dependent α-ketoglutarate dehydrogenase (KGDH) enzyme complex in brain in TD (Gibson *et al*., 1984; Butterworth *et al*., 1986) and in patients with WE (Butterworth *et al*., 1993). This enzyme plays a key role in the regulation of glucose metabolism, with a reduction in its activity being a likely contributor to the decrease in energy status in affected areas of the brain. However, the exact cause of the regionally and cellularly distinctive pattern of neurodegeneration remains unclear.

Over the years, evidence indicates that oxidative stress, glutamate-mediated excitotoxicity and inflammation are major contributors to the resulting neuropathology in TD. This review will discuss the evidence for this, and the role of these three processes in the pathophysiology of this brain disorder.

ROLE OF OXIDATIVE STRESS

Nitric oxide synthase in TD

Increased production of reactive oxygen species (ROS) has been reported in brain in TD (Langlais *et al*., 1997), and oxidative stress is related to the pathology in human WE as evidenced by increased neuronal peroxidase activity. Changes consistent with oxidative stress in TD include findings of increased expression of heme oxygenase (HO-1) and ICAM-1 (Gibson and Zhang, 2002) as well as microglial activation (Todd and Butterworth, 1999). Moreover, selective induction of the endothelial isoform of nitric oxide synthase (eNOS) has been reported in the brains of TD animals (Fig. 1), although an overall decrease in activity of total NOS (eNOS, iNOS, and nNOS isoforms) has been described in vulnerable regions in TD animals (Rao *et al*., 1996), mainly due to the neuronal cell death. Increased eNOS protein was particularly evident in the medial thalamus in TD; no significant changes were observed in the cerebral cortex. These findings suggest that an early, region-selective insult in TD is associated with increased production of nitric oxide (NO) by cerebrovascular endothelial cells. Using an immunohistochemical approach, increased eNOS immunostaining was observed in thalamic microvessels of TD mice (Calingasan and Gibson, 2000) and increased immunostaining of iNOS in microglia was also reported in the brains of these animals. These findings of increased eNOS expression and NO production are likely to be of pathophysiologic significance since NO can react with the superoxide radical (O_2^-) to form peroxynitrite ($ONOO^-$), which is toxic to neurons. The toxicity results from its ability to nitrate tyrosine residues and to

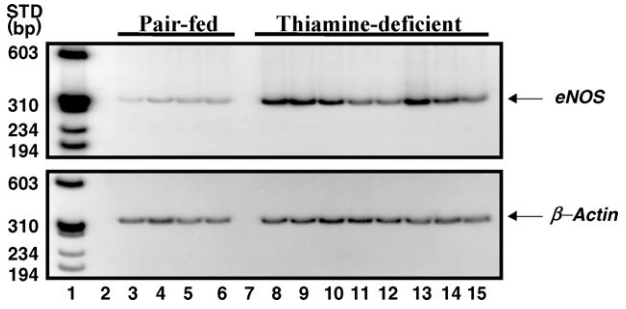

Fig. 1 Increased expression of endothelial nitric oxide synthase (eNOS) mRNA in the medial thalamus of thiamine-deficient rats and pair-fed controls. Total RNA was extracted from the medial thalamus of thiamine-deficient rats or from pair-fed controls. β-Actin (347bp) and eNOS (351bp) were reverse-transcribed and amplified by PCR for 18 and 32 cycles, respectively. Lane 1: molecular weight standards; lanes 3–6: pair-fed controls; lanes 8–15: symptomatic thiamine-deficient rats; lanes 2 and 7: AMV reverse transcriptase was omitted as a negative control. Reproduced from Kruse *et al*. (2004), with permission.

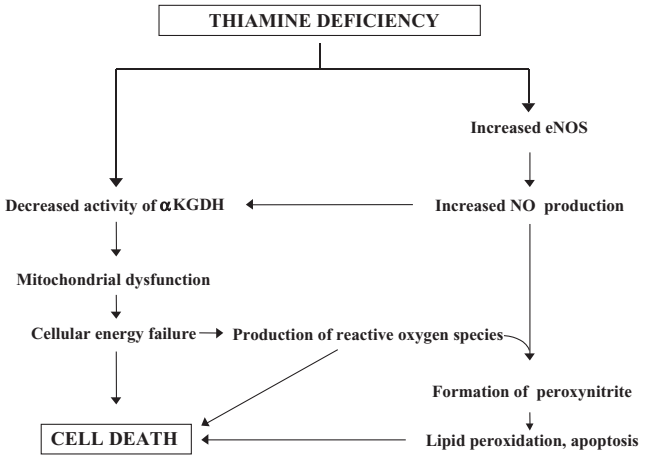

Fig. 2 Possible mechanisms whereby eNOS induction could participate in the pathogenesis of selective neuronal loss due to thiamine deficiency.

cross-link thiol groups in proteins. Indeed, increased nitrotyrosine immunolabeling has been described in neuronal processes in thalamus of TD animals (Calingasan *et al*., 1998), and may be indicative of its involvement in the pathogenesis of selective neuronal cell death due to TD. Studies have also demonstrated that targeted disruption of the eNOS gene results in a reduction in the extent of the necrotic lesions in the thalamus of TD animals (Calingasan and Gibson, 2000), further evidence in support of an involvment of the gene in the pathophysiologic consequences of this disorder.

Oxidative stress and KGDH

Increases in NO are asociated with decreased activities of α-KGDH (Gibson *et al*., 1999). Moreover, an inhibitory effect of NO on cytochrome c oxidase activities has been reported in both isolated mitochondria and in neuronal preparations (Brown, 2001). Based upon these observations, it is likely that NO and peroxynitrite-mediated mitochondrial dysfunction and damage play a role in the pathogenesis of TD-related neuronal cell loss. Moreover, evidence in favor of such a mechanism in relation to neurodegeneration in general is compelling. For example, thiamine-dependent enzymes including KGDH are reduced in autopsied brain tissue from patients with Alzheimer's disease. NO exerts an inhibitory effect on cytochrome c oxidase and KGDH activities in microglia (Park *et al*., 1999), and both microglial activation and reductions in KGDH have consistently been described in Alzheimer's disease (Gibson and Zhang, 2002), ischemic brain injury (Cao *et al*., 1988) and WKS (Butterworth *et al*., 1993). In the case of WE, these mechanisms constitute a vicious cycle whereby TD results in a reduction in activity of KGDH, which in turn results in mitochondrial uncoupling. Simultaneously, NO produced by activation of endothelial and microglial NOS combines with the superoxide radical (O_2^-) to form peroxynitrite, as previously mentioned, both of which result in mitochondrial dysfunction and additional reductions in activities of KGDH (Fig. 2).

Further evidence in support of a role of oxidative stress in the pathogenesis of selective neuronal loss in TD is provided by the report of a neuroprotective effect of L-deprenyl (Todd and Butterworth, 1998), an agent with potent oxygen free radical scavenging properties. In addition, a preliminary report suggests that superoxide dismutase is increased in vulnerable brain regions and was reduced following treatment with L-deprenyl (Todd and Butterworth, 1997), while treatment with the antioxidant *N*-acetylcysteine protected against neuronal cell death (Hazell and Wang, 2005). Interestingly, L-deprenyl has been shown to cause a slowing in the evolution of the neurological deficits in Alzheimer's disease (Schneider *et al*., 1991), as well as Parkinson's disease (Oakes, 1993) another neurodegenerative disorder associated with decreased activities of KGDH.

Oxidative stress and the blood-brain barrier

Numerous studies in the past indicate that breakdown of the blood-brain barrier (BBB) occurs in TD (Warnock and Burkhalter, 1968; Manz and Robertson, 1972; Watanabe, 1978; Harata and Iwasaki, 1995). Indeed, reports of increased endothelial transport being a contributor to BBB disruption in TD (Calingasan *et al*., 1995; Harata and Iwasaki, 1995) suggest that the functional integrity of these cells may be impaired. NO has the ability to disrupt the BBB (Au *et al*., 1985); since endothelial cells and astrocytes are important sources of NO (Murphy *et al*., 1990; Moncada *et al*., 1991), and free radicals are capable of increasing endothelial permeability in brain (Chan *et al*., 1984) and other tissues (Sacks *et al*., 1977; Del Maestro *et al*., 1980), it is likely that oxidative stress plays a role in BBB disruption. Indeed, previous studies have reported the presence of iron deposits in TD brain, in the form of both iron and ferritin (Calingasan *et al*., 1998, 1999), evidence of both BBB disruption and oxidative processes at work.

GLUTAMATE-MEDIATED EXCITOTOXICITY

The evidence for an excitotoxic event

Previous studies have provided strong evidence for the existence of excitotoxic-mediated cell death in TD. Decreased incorporation of [^{14}C]-glucose into glutamate (Gaitonde *et al*., 1975) is consistent with reduced activities of KGDH, resulting in reduced energy status in TD animals (Aikawa *et al*., 1984). In addition, treatment with the noncompetitive NMDA glutamate

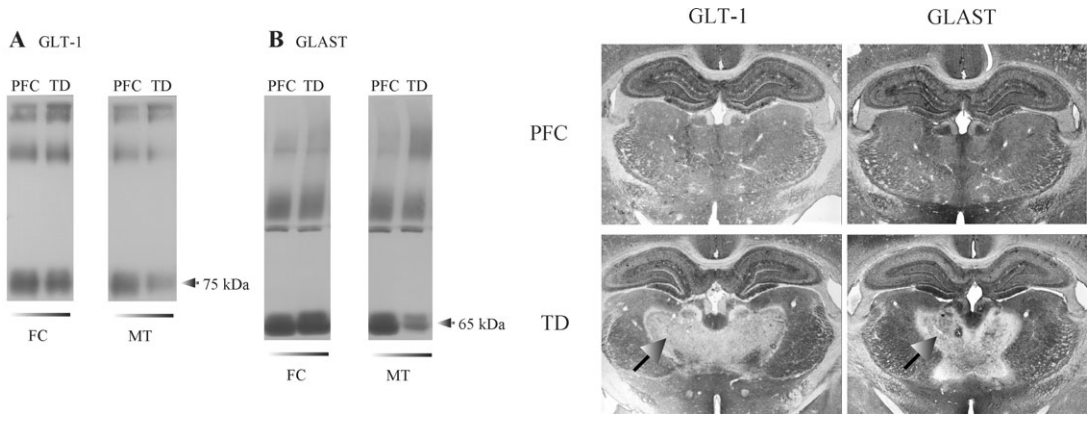

Fig. 3 Downregulation of the astrocytic glutamate transporters GLT-1 and GLAST in thiamine deficiency (TD). Immunoblotting shows loss of GLT-1 and GLAST in the medial thalamus (MT) of TD animals compared to pair-fed controls (PFC). The non-vulnerable frontal cortex (FC) showed no changes in transporter levels. Immunohistochemical staining of coronal sections at the level of the posterior thalamus indicates a loss of GLT-1 and GLAST immunoreactivity in the medial thalamic area (arrows). Adapted from Hazell *et al*. (2001).

receptor antagonist MK-801 leads to a reduction in the extent of neuronal damage in thiamine-deficient rats (Langlais and Mair, 1990). Focal increases in the extracellular glutamate concentration restricted to vulnerable brain regions in TD (Hazell *et al*., 1993; Langlais and Zhang, 1993) provided the first direct evidence for glutamate-mediated excitotoxicity, along with the presence of excitotoxic-like lesions in damaged areas of the brain (Armstrong-James *et al*., 1988). Indeed, the ultrastructural appearance of the affected thalamus (Watanabe, 1978) is also similar to that seen in excitotoxic-mediated necrosis (Olney, 1978). Downregulation of the astrocytic glutamate transporters GLT-1 (Glutamate Transporter 1) and GLAST (Glutamate/Aspartate Transporter) was later reported to be localized to vulnerable brain regions in TD (Hazell *et al*., 2001) (Fig. 3). These astrocytic transporters provide the major spatial buffering of extracellular glutamate levels in brain (Danbolt, 2001). *In vitro* findings of a loss of the GLAST protein along with decreased glutamate transport kinetics in an *in vitro* astrocyte model of the disorder (Hazell *et al*., 2003) add further credence for implication of a loss of glutamate transporter function in the pathophysiology of TD. In addition, oxidative stress can rapidly lead to inhibition of glutamate transport due to transporter protein nitrosylation by peroxynitrite formation (Volterra *et al*., 1994; Trotti *et al*., 1996, 1998; Agostinho *et al*., 1997). Together with earlier reports of a reduction in Ca^{2+}-dependent release of glutamate from hippocampal slices in TD (Lê *et al*., 1991), these studies indicate that the source of increased extracellular glutamate may be mainly due to loss of glutamate transporter function rather than increased release of neurotransmitter glutamate from the presynaptic terminal. On the other hand, recent studies indicate that levels of complexins, presynaptic terminal proteins that play an important role in the regulation of neurotransmitter release (Pabst *et al*., 2000; Tang *et al*., 2006) are downregulated in vulnerable brain regions in TD (Hazell and Wang, 2005) that may reflect either enhanced release of glutamate and/or GABA or an inhibition of glutamate/GABA release by neurons. Cpx I is expressed in axosomatic (inhibitory) synapses, while Cpx II is localized in axodendritic and axospinous synapses (Yamada *et al*., 1999), of which the majority are excitatory (McMahon *et al*., 1995). This effect on complexin levels is reversible with antioxidants such as *N*-acetylcysteine (Hazell and Wang, 2005), suggesting an involvement of oxidative stress. Further studies are required to establish the exact significance of these changes in complexin levels in TD.

Depolarization and immediate-early gene expression

Sustained depolarization is a typical feature of overexcitation of neural tissue. Expression of the immediate-early genes (IEGs) c-*fos*, c-*jun*, *fos*-B and NGFI-A (egr-1) is dramatically increased in, and localized to, the thalamus and inferior colliculus in TD in advance of the appearance of necrotic lesions (Hazell *et al*., 1998a). An earlier increase in c-*fos* expression was also described in the same report, consistent with the initiation of a pre-symptomatic apoptotic event, with previous studies reporting the existence of apoptosis in TD (Matsushima *et al*., 1997). IEGs encode transcriptional regulating factors that are induced following depolarization (Morgan and Curran, 1986; Bartel *et al*., 1989; Sheng *et al*., 1990). Evidence of sustained depolarization was provided in a study in which increased activation of L-type voltage-sensitive calcium channels (VSCCs) was described in TD, and which was reversible following thiamine replenishment (Hazell *et al*., 1998b) (Fig. 4). The L-type VSCCs are activated principally by depolarization of the cell membrane. In this activated state, allosteric changes in these channels facilate the entry of Ca^{2+} along its concentration gradient into the cell. IEGs are also implicated in the control of genes that mediate both apoptosis and excitotoxic-induced cell death (Colotta *et al*., 1992; González-Martín *et al*., 1992; Dragunow *et al*., 1993; Estus *et al*., 1994). Furthermore, activation of L-type VSCCs has been linked to the induction of IEGs (Morgan and Curran, 1986; Murphy *et al*., 1991), as well as to the production of trophic factors (Zafra *et al*., 1990) that may also have a role to play in the pathogenesis of neuronal cell death in TD. These findings suggest that increased expression of IEGs may be a major contributing factor in excitotoxicity in TD. Further investigations into the role of these transcription factors are warranted.

Cerebral energy metabolism and lactic acidosis

Decreased activity of the rate-limiting enzyme KGDH in TD represents a major probable cause of decreased energy status in

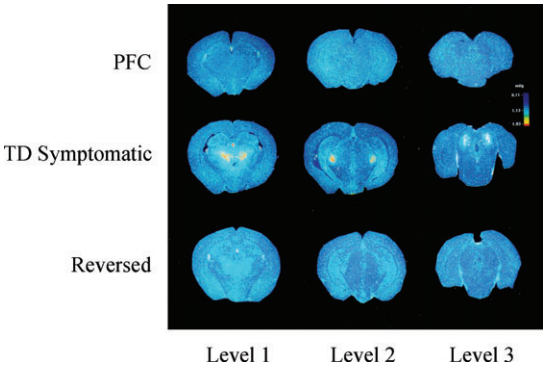

Fig. 4 Thiamine deficiency leads to activation of L-type voltage-sensitive calcium channels (VSCCS) in brain. Representative coronal sections from a pair-fed control (PFC), thiamine-deficient rat at loss of righting reflex (TD symptomatic), and thiamine-replenished (Reversed) animal showing increased specific binding of [^{3}H]-nimodipine, an L-type VSCC antagonist, at the level of the vulnerable thalamus (level 1) including the medial geniculate (level 2) and the inferior colliculus (level 3). Specific binding of nimodipine increases due to activation of L-type VSCCs. The activated channel state occurs as a consequence of membrane depolarization. Reproduced from Hazell *et al*. (1998b), with permission.

vulnerable areas of the brain, with impaired glucose metabolism being a crucial factor contributing to loss of neuronal function, including glutamatergic neurotransmission. This is consistent with the findings of Robertson and colleagues (1975) in which levels of incorporation of [^{14}C]-3-*O*-methyl-D-glucose were depressed in the vulnerable areas of the brain in TD, indicating decreased glucose uptake. These findings were reinforced by studies that showed dramatically reduced local cerebral glucose utilization (Hakim and Pappius, 1981, 1983) and decreased levels of ATP and phosphocreatine (Aikawa *et al*., 1984). However, the exact relationship between KGDH enzyme activities and focal vulnerability remains uncertain since decreased activities in brain occur in animals in the absence of any change in the concentration of protein, with neither activity nor protein levels being predictive of vulnerability (Sheu *et al*., 1998).

In TD, a major consequence of impaired oxidative metabolism is the focal accumulation of lactic acid in the brain, a phenomenon that has been well recognized for many years (Kinnersley and Peters, 1930; Holowach *et al*., 1968; McCandless and Schenker, 1968). Lactic acidosis results in tissue and cellular acidification *in vivo* (Mutch and Hansen, 1984) with previous studies indicating that exposure of astrocytes to lactic acid results in impaired glutamate transport function (Bender *et al*., 1997). Since increased lactate and the resulting acidosis have been shown to be localized in regions that subsequently develop histological lesions in TD (McCandless, 1982; Hakim, 1984; Munujos *et al*., 1993; Navarro *et al*., 2005), it is possible that lactic acidosis plays a significant role in the development of excitotoxic-mediated damage in this disorder.

Brain edema and lactic acidosis

In WE, previous studies have established that cerebral vulnerability is associated with the presence of edema, with studies of ante-mortem brain tissue using magnetic resonance imaging (MRI) reporting the presence of signal hyperintensities in focal areas of damage (Bergui *et al*., 2001) consistent with the presence of increased water content. Case studies have also shown reversibility of such MRI hyperintensities and lowered densities on computerized tomography following thiamine administration, suggesting that edema may be an important underlying factor in many of the neurological abnormalities occuring in these patients (McDowell and Leblanc, 1984; Bergui *et al*., 2001). Although the exact cause of this edema is unclear, the considerable evidence for BBB disruption in TD indicates that vasogenic edema is a major cause of the consequential brain tissue swelling.

Apart from vasogenic edema, cytotoxic edema represents the other major process that can lead to brain swelling. Extra- and intracellular acidosis are associated with cytotoxic edema (Myers, 1979; Kalimo *et al*., 1981; Pulsinelli *et al*., 1982; Jenkins *et al*., 1984), and lactic acidosis is a major consequence of TD; thus, lactate production is likely to lead to cytotoxic edema. Since astrocytes are the principal source of lactate production (Pellerin *et al*., 2007), and glial swelling is an important feature of TD (Collins, 1967; Robertson *et al*., 1968; Watanabe and Kanabe, 1978; Watanabe *et al*., 1981), it is probable that lactate-induced swelling in astrocytes is also a contributing factor to the brain edema observed in human cases of WE. Such astrocyte swelling is also likely to play a role in glutamate-mediated excitotoxicity since previous studies have established that swelling of these cells results in the release of glutamate (Kimelberg *et al*., 1995), which may have pathological implications in terms of interstitial glutamate levels. In addition, recent studies have demonstrated that TD in cultured astrocytes leads to swelling of these cells that is associated with alterations in the levels of aquaporin-4 (AQP-4), a major water channel protein localized predominantly in brain (Chan *et al*., 2004). Thus, lactic acidosis may mediate these changes in AQP-4 expression that lead to swelling of the astrocytes in TD with pathological consequences. Indeed, recent evidence supports this contention whereby lactic acid increases AQP-4 gene expression in astrocytes (Morishima *et al*., 2008), while focal accumulation of lactate and resulting pH changes in brain have been suggested as a possible cause of neuronal cell death in TD (Hakim, 1984).

Astrocytes, K^+ spatial buffering and glutamate release

In addition to the uptake of glutamate, astrocytes perform another important role of clearance of K^+ from the extracellular space (Hertz, 1965). In TD, overstimulation of neurons by glutamate results in a rise in extracellular K^+ that may also contribute to sustained depolarization. Although astrocytic spatial buffering of K^+ is performed mainly by entry of K^+ into the cells via passive currents with subsequent propagation through the glial syncytium via gap junctional communication (Kuffler *et al*., 1966; Karwoski *et al*., 1989), higher extracellular K^+ levels due to increased neuronal activity can lead to the uptake of K^+ via activity of the astrocytic Na^+-K^+-ATPase (Walz and Hertz, 1982). Such spatial buffering of external K^+ by astrocytes can also cause their swelling (Hansson *et al*., 1994), possibly contributing to swelling-induced release of glutamate (Kimelberg *et al*., 1990, 1995), and therefore a further potential source of glutamate-mediated excitotoxicity. Since glial swelling and brain edema are features of TD, it is conceivable that K^+-mediated astrocytic swelling may contribute to

excitotoxic-mediated damage in TD by stimulating the release of glutamate from these cells.

INFLAMMATORY RESPONSES

Cerebral inflammation is now recognized as a key component of several neurological diseases including stroke, multiple sclerosis and Alzheimer's disease, along with other conditions such as brain trauma. During the 1960s, alterations in glial cell morphology in TD including evidence of swelling and the appearance of phagocytic vacuoles (Collins, 1967; Robertson *et al*., 1968) were first reported. These findings are consistent with pathological changes that can be attributable to the presence of an inflammatory process. More recently, further evidence has been described in support of this mechanism in TD; increased microglial reactivity, an indication of inflammation, is an early cellular response, while production of pro-inflammatory cytokines in both vulnerable and non-vulnerable regions of brain have been reported (Ke *et al*., 2006; Vemuganti *et al*., 2006; Karuppagounder *et al*., 2007). In particular, using a microarray approach (Vemuganti *et al*., 2006), it was possible to demonstrate that in vulnerable brain regions in TD, inflammatory genes represent the largest functional group of transcripts upregulated. These include the pro-inflammatory cytokines (IL-6, IL-18, TNF-α, AIF1 and osteopontin), chemokines (MCP-1, MIP-1α, MIP-1β and Gro1), interferons (IFNs) and IFN-inducible proteins. Interestingly, many transcription factors known to control inflammatory gene expression (Egr-1, c-EBP-β, c-EBP-δ, CPBP and Klf-4) were also upregulated following TD. Of these, Egr-1 and c-EBP-β may play an important role in starting the inflammatory cascades following oxidative impairment. Levels of these various inflammatory-related gene products in different brain regions may be an important factor(s) in the determination of selective vulnerability in TD.

CONCLUSIONS

TD results in an impairment of oxidative metabolism. The consequences of this include a series of events that set the stage for cerebral vulnerability. Here we have highlighted oxidative stress, excitotoxicity and inflammation in terms of our present understanding of their involvement in TD. How these processes together help determine focal neuronal cell loss in TD and in cases of WE remains unresolved at the present time. However, what is now clear is that TD represents a useful model system for examining the interrelationships between these different mechanisms, as impaired oxidative metabolism is also a feature of neurodegenerative disease, and we know that several of these disease states also display elements of oxidative stress, excitotoxicity and inflammation. Thus, TD represents an excellent model for studying the interrelationships between these different mechanisms, with the potential to provide new insight pertaining to the pathophysiology of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and other disease conditions in which these three processes are known to occur.

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