Amino Acids (2003) 25: 427–436
DOI 10.1007/s00726-003-0028-6

Amino Acids
Printed in Austria

Relevance of protein nitration in brain injury: a key pathophysiological mechanism in neurodegenerative, autoimmune, or inflammatory CNS diseases and stroke

Review Article

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Received December 2, 2002
Accepted May 8, 2003
Published online August 21, 2003;

Summary. This review has focused on the evidence for the involvement of nitrative oxidation in certain neurodegenerative disorders (Parkinson's Disease, Alzheimer's Disease, Amyotrophic Lateral Sclerosis), stroke, and inflammatory and autoimmune disorders (with particular attention devoted to multiple sclerosis).

The relationship between protein peroxidation and pathological changes observed in the above disorders has been reported. Whereas many of the findings are from studies with animal models and autoptic specimens from human patients, few data are available from cerebrospinal fluid and blood samples of the patients at different times and disease stages.

The participation of nitrative oxidation to the direct and indirect injury of neurons and other cells of the brain (i.e., oligodendrocytes, for multiple sclerosis) is clear; less evident is their relevance for the development and progression of these disorders.

Further studies should be aimed to establish the clinical and prognostic value of peroxidative markers for the CNS diseases considered. This is fundamental for the development of therapeutic interventions antagonizing nitric oxide-related species damage.

Keywords: Protein nitration – Nitrotyrosine – Neurodegenerative diseases – Inflammatory and autoimmune diseases of the central nervous system – Stroke – Pathogenic mechanisms

Introduction

Cellular stress is considered one of the pivotal mechanisms involved in the initiation and progression of neuronal cell injury underlying several central nervous system (CNS) disorders. Mediators of cellular stress and damage are reactive oxidative species (ROS) such as hydroxyl radicals ($OH^{\bullet}$), tyrosyl radicals ($TyrO^{\bullet}$) and reactive nitrogen species (RNS) including peroxynitrites (PN), nitric oxide ($NO^{\bullet}$) and nitric dioxide ($NO_2^{\bullet}$) radicals. Their increased production together with the lack or insufficiency of antioxidant capacity have been hypothesized and at least in part demonstrated to underlie different neurodegenerative disorders. Biochemical events initiated by these reactive species can induce lipid peroxidation and protein oxidation and nitration, leading to cell damage and death by apoptosis and autoschizis (Kochman et al., 2002).

In the last few years it has become evident that tyrosine and $TyrO^{\bullet}$ are implicated in nitrative and oxidative events involved in neuronal death. Both can interact with ROS and RNS via radical and chain propagating reactions.

Oxidation of tyrosine by the $SOD1/H_2O_2/HCO_3^-$ system is the basis of major reaction pathways leading to the formation of dityrosine (DT), 3-nitrotyrosine (3-NT), NO_2-DiTyr (3-NDT) and other higher oxidation products. In particular, 3-NDT has been proposed as a reliable marker of both oxidation/nitration of Tyr *in vivo*. However, the relationship between 3-NT bound to proteins and free 3-NT is still unknown, as well as its biological and pathological relevance (Halliwell, 1997; Zhang et al., 2000; see also Duncan, this volume).

Nitration of Tyr residues has important consequences for the cell, by inducing structural (i.e., in neurons it may disrupt neurofilaments) and enzymatic alterations (Beckman and Koppenol, 1996); and because it inhibits tyrosine

phosphorylation and thereby interferes with important signaling pathways (Van der Vliet et al., 1995).

Elderly people are more susceptible to oxidative and nitrative stress and damage than the young. An age-related impairment for recognition and turnover of modified cell components, particularly proteins, exists and antioxidant mechanisms are also less efficient (Gracy et al., 1999). Hence, oxidative/nitrative damage could play a causative role in many chronic-degenerative diseases of aging, including neurodegeneration.

In particular, the role of nitrotyrosylation in pathogenic mechanisms seems to occur not only in neurodegenerative disorders such as Parkinson's Disease (PD), Alzheimer's Disease (AD) and Amyotrophic Lateral Sclerosis (ALS), but also in ischemic stroke, and inflammatory and autoimmune demyelinating diseases, particularly multiple sclerosis (MS) (Kochman et al., 2002). This review will be focused on the evidence available until now from experimental models and patients affected.

Parkinson's Disease (PD)

PD results from degeneration of dopaminergic neurons in the substantia nigra. There is considerable evidence that oxidative stress is associated with the progression of sporadic PD and may be an early stage in its progression (Sian et al., 1994; Beal, 2002; Jenner and Olanow, 1998). One of the mechanisms of oxidative dopaminergic cell injury is the nitration of protein residues mediated by PN, the reaction product of NO and superoxide radicals. In particular, formation of RNS such as PN has been implicated in apoptotic dopaminergic cell death in PD (Beal, 2002).

The presence of nitrotyrosine immunoreactivity has been demonstrated in Lewy bodies, which are a characteristic feature of PD and of dementia with Lewy body disease. This increased nitration of Tyr residues has been shown especially in the protein neurofilaments of the core (Good et al., 1998). This observation provides a definite link between oxidative stress and excitotoxicity within the vulnerable neurons of PD. Further evidence for the enhanced protein Tyr nitration in PD comes from studies showing that polymorphonuclear cells of PD patients exhibit an increase in rate of NO production that is accompanied by accumulation of nitrotyrosine-containing proteins together with an over-expression of neural nitric oxide synthase (nNOS) (Gatto et al., 2000).

In methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice elevated levels of DT and both bound and free 3-NT were found in the striatum and ventral midbrain (Pennathur et al., 1999), lending additional support to the involvement of nitration of Tyr residues in PD.

It has also been demonstrated that the two neurotoxins, 1-methyl-4-phenylpyridinium (MPP+) and 6-hydroxydopamine, lead to Tyr nitration via tyrosine hydroxylase (TH) in cultured dopaminergic neurons (Pong et al., 2000). Nitration of Tyr residues is responsible for inactivation of TH, which is the rate-limiting enzyme in the synthesis of catecholamines with a consequent failure in the synthesis of dopamine. Nitrotyrosine formation on TH moieties seems to parallel the decrease of dopamine levels in the striatum and this mechanism has also been proposed to explain the increased severity of PD (Ara et al., 1998). Tyr residues are also essential in monoamine oxidase (MAO) substrate and inhibitor specificity. Therefore, their nitration could affect dopamine and melanin metabolism (Geha et al., 2000).

The presence of mutated α-synuclein found in some cases of familial PD results in self-aggregates and accumulation of abnormal proteins in the cells to give insoluble deposits that cannot undergo proteolysis by the proteasomes (Polymeropoulos et al., 1997; Mezey et al., 1998). This increases the susceptibility to oxidative stress, which is reflected by decreased levels of glutathione (GSH) and increased levels of 8-hydroxyguanine (8-OHG), protein carbonyls, lipid peroxidation markers and also 3-NT. Paradoxically, these oxidant stress-related changes may further promote aggregation of α-synuclein (Hashimoto et al., 1999) and may facilitate enhancement of the susceptibility to other injuries, including the inhibition of complex I in mitochondria by the inhibitor MPP+, proteasome inhibition by lactacystin and protein kinase inhibition by staurosporine. This multi-level challenge to neuronal cells associated with oxidative damage has been reported to trigger apoptosis (Lee et al., 2001a).

Abnormal staining of α-synuclein is not specific for familial PD, but has also been found in neurofilament inclusions in multisystemic atrophy (Spillantini et al., 1998) and AD (Goedert et al., 1998), and this could contribute to the increased neuronal vulnerability in these pathological conditions.

Alzheimer's Disease (AD)

Cognitive decline and dementia are key features of AD that results from degeneration of neuronal function in specific brain regions.

AD brain exhibits region-specific patterns of amyloid deposition, neurofibrillar tangles accumulation and neuronal death. The limbic system and associated areas in the

neocortex show the most pronounced histopathological alterations in AD, whereas cortical somatosensory and cerebellar neurons are relatively unaffected. Experimental models of AD attempt to link disease progression with an inflammatory component combined with increased oxidative stress (Rogers et al., 1996).

Enhanced oxidative stress in the AD brain is manifested by increased protein carbonyl content, lipid and DNA oxidation products, and inactivation of sensitive enzymes (Smith et al., 1991; Balazs and Leon, 1994; Chen et al., 1994; Hensley et al., 1995; Lovell et al., 1995; Smith et al., 1996, 1997; Butterfield et al., 1997; Lyras et al., 1997; Sayre et al., 1997).

These oxidative events seem to appear early in the process of neurodegeneration in AD (Numomura et al., 2001). NO-derived species are significant contributors to the pathogenic mechanisms underlying AD. Affected neurons demonstrate indices of nitro-oxidative stress and immunoreactive NOS suggesting that NOS-modified neurons are implicated in AD (Williamson et al., 2002).

In a study by Hensley et al. (1998) carried out on specimens of AD brains, DT and 3-NT levels were consistently elevated in the hippocampus and neocortical regions, and in ventricular fluid (VF), reaching values five to eightfold greater than in the brain and VF of normal subjects. The PN scavenger uric acid was, on the contrary, reduced in the same areas.

In a study by Tohgi et al. (1999a), the 3-NT concentration and the 3-NT/tyrosine ratio were also increased (>sixfold) in the cerebrospinal fluid (CSF) of AD patients compared to age-matched controls without cognitive impairment. Moreover, there was a significant correlation between the higher levels of these markers and the decrease in cognitive function.

Neurofibrillar tangles and dysfunctional neurons frequently display high levels of 3-NT or other markers of nitrosative stress and immunoreactive NOS (Colton et al., 2002).

On the other hand, neuronal DNA damage without evidence of tangle formation is associated with up-regulation of 3-NT in AD (Su et al., 1997). Neurotoxicity of beta-amyloid (Abeta)-42, which accumulates extracellularly in senile plaques, has been well established. It is associated with a larger area of glial fibrillary acidic protein immunoreactivity and a greater density of reactive astrocytes than is Abeta-40 (Klein et al., 1999).

Immunohistochemical staining for markers of oxidative and nitrative stress, such as 8-hydroxydeoxyguanosine (8-OHdG) and 3-NT, is significantly more intense around Abeta-40.

Although a strict correlation between oxidation and inflammatory biomarkers has not been definitively established for the AD brain, the inflammation-like pattern of AD brain oxidation suggests that inflammatory events are involved. In particular, it is known that activation of microglia leads to the release of both superoxide and hydrogen peroxide and that in the presence of β-amyloid peptide or proinflammatory cytokines astroglia and microglia express inducible NOS (iNOS) and generate NO-derived species, including PN (Colton et al., 1994; Beckman et al., 1994; Goodwin et al., 1995; Ii et al., 1996; Hensley et al., 1997).

In a recent study, Luth et al. (2002) observed a high expression of both iNOS and endothelial NOS (eNOS) in glial cells and astrocytes. This was accompanied by an aberrant expression of nNOS in affected areas exhibiting increased formation of 3-NT. This suggests that the increased expression of all NOS isoforms in astrocytes, glial cells and neurons contributes to the synthesis of PN. In view of the wide range of isoform-specific NOS inhibitors available in the near future for therapeutic use, the identification of the most important isoform responsible for the formation of NO-derived species, and particularly PN in AD, could be of exquisite relevance in treating AD.

Amyotrophic lateral sclerosis (ALS)

ALS is a neurodegenerative disease occurring in adulthood that is characterized by the progressive loss of motor neurons in the motor cortex, brainstem and spinal cord. This results in progressive muscular weakness and eventually death within 3–5 years from the onset (Wong et al., 1995). About 10% of ALS cases are familial (FALS) and at least 80 mutations of the gene encoding for the isoform 1 of the enzyme Cu/Zn-superoxide dismutase (SOD1) have been identified in 20% of ALS patients (particularly familial cases) (Brown, 1995; Aguirre et al., 1998).

The majority of the mutations identified in SOD1 protein are located outside the catalytic site of the enzyme with a variable effect on the superoxide-scavenging activity (Swingler et al., 1995). Over-expression of mutated Cu/Zn-SOD1 is responsible for ALS-like symptoms in transgenic mice (Gurney et al., 1994). In particular, transgenic mice that over-express G93A and G37R mutant SOD1 protein suffer severe loss of motor neurons (Ferrante et al., 1997a; Liu et al., 1999).

A recent immunohistochemical study carried out on transgenic mice with G93A mutant SOD1 gene showed, from the early symptomatic to the end stage, positive iNOS and 3-NT immunoreactivity in proliferated reactive astrocytes as well as somata of the anterior horn cells

(Sasaki et al., 2001). The contribution of reactive astrocytes to the death of motor neurons in ALS via peroxynitrite-dependent damage is also suggested by the finding that these cells respond to extracellular PN by developing morphologic characteristics resembling those of process-bearing cells displaying intense glial fibrillar acidic protein and iNOS immunoreactivity. This phenotypic change results in broad cytotoxicity for motor neurons and was largely prevented by either NOS inhibitors or peroxynitrite scavengers, but not by trophic factors. Neuronal death was associated with highly positive staining for activated caspase-3 and 3-NT (Cassina et al., 2002).

Furthermore, SOD with different FALS mutations generated hydroxyl radicals more readily than wild-type SOD1 (Wiedau-Pazos et al., 1996; Yim et al., 1996).

Even in *in vitro* experiments, mutant SOD1 accelerates cell death (apoptosis), whereas expression of wild-type SOD1 protein protects cells against oxidative stress insults including protein Tyr nitrosylation (Ghadge et al., 1997; Rabizadeh et al., 1995). The effect of mutant SOD1 seems to be attenuated by the antioxidant-related and anti-apoptotic product of the oncogene Bcl-2 (Lee et al., 2001b).

Several lines of evidence suggest that oxidative stress is involved in the pathophysiology of ALS. They include reduced levels of GSH and increased levels of oxidative damage products such as protein carbonyls, 8-OHdG and 4-hydroxy-2-*trans*-nonenal (HNE) (Lanius et al., 1993; Shaw et al., 1995; Ferrante et al., 1997b; Hall et al., 1998; Pedersen et al., 1998). In ALS the attack of RNS on protein has been confirmed by the finding of increased levels of 3-NT in autoptic cases of both FALS and sporadic ALS (SALS) (Abe et al., 1997; Halliwell, 1997; Beal et al., 1997; Crow et al., 1997). A study carried out by Tohgi et al. (1999b) provided *in vivo* evidence for increased nitration of Tyr residues in patients with SALS, which is in line with previous findings of PN together with stable NO metabolites, i.e., nitrates and nitrites, in the CSF (Tohgi et al., 1999c, Shaw e Williams, 2000). As free Tyr concentrations in patients with SALS were not reduced, the increase in 3-NT in the CSF has been attributed to both an increase in nitrated Tyr in proteins and increased degradation of 3-NT-containing proteins, these being highly vulnerable to proteolytic degradation.

Nevertheless, results of other studies indicate that tyrosine nitration is not a major factor in the toxicity of G93A mutant SOD1. In a primary cell culture model, the prevention of 3-NT formation by inhibiting NOS rescued motor neurons from excitotoxic death but did not delay the death of motor neurons expressing G93A mutant SOD1 (Doroudchi et al., 2001).

The mechanisms by which PN selectively mediates motor neuron death in SALS, in which mutation-related gain of function of the SOD gene is not usually found, remain to be established.

Stroke

NO and oxygen free radicals have been proposed to be involved in the cascade of injury elicited by ischemia and reperfusion-mediated brain injury. Superoxide generation is augmented by ischemia reperfusion injury (Forman et al., 1998) and also a greater amount of NO is produced (Bidmon et al., 1998). Therefore, it is obvious that the brain expresses all the elements for the formation of PN in cytotoxic amounts, and consequently is an elective target tissue for NO-induced damage under stress conditions (Gürsoy-Ödzemir et al., 2000; Hirabayashi et al., 2000).

The involvement of NO release, derived from both up-regulation of constitutive and iNOS as well as nitrative injury, has been demonstrated in animal models of stroke.

In stroke-prone spontaneous hypertensive rats, endothelial dysfunction is associated with increased staining for 3-NT in vessels, and this can be attenuated by antioxidant treatment (Ma et al., 2001; Leker et al., 2002).

In the spontaneous stroke model immunohistochemical staining of 3-NT was also detected around vessels and in neuronal cells of brain ischemic lesions. This was observed immediately following the fluctuation of serum NO(x) at the onset of stroke (Tabuchi et al., 2002).

Concurrent formation of PN with the expression of iNOS has been demonstrated in the penumbra of rat brain ischemic lesions during reperfusion following middle cerebral artery occlusion and also in human cerebral infarcts (Forster et al., 2001). The increased NOx levels observed in the circulation have been attributed to the over-expression of iNOS (Suzuki et al., 2002). It was postulated that nNOS contributes to PN formation observed in the cortex during the early phase of reperfusion (Rodrigo et al., 2001).

The clear relationship between nNOS over-expression and peroxynitrite production was established in a recent investigation of the effects of oxygen and glucose deprivation on the distribution of nNOS and iNOS and protein nitration in the rat cerebral cortex, in a model of global cerebral ischemia and reperfusion. In this study, cerebrocortical injury, as shown by diffusion magnetic resonance imaging (MRI) was accompanied by increasing morphological changes in the large type I interneurons expressing nNOS and by the appearance of nNOS in small type II neurons. This NOS isoform showed an initial increase

followed by a fall 6 hours after reperfusion. Inducible NOS immunoreactivity appeared in neurons, especially the pyramidal cells of layers IV–V after 4 hours of reperfusion with a concomitant increase in calcium-independent NOS activity. During reperfusion, immunoreactive 3-NT present in the nuclear regions and perinuclear cytoplasm in control animals showed a widespread increase with a concurrent time-dependent translocation to the cytoplasm and neural processes (Alonso et al., 2002). This immunohistochemical appearance concurs with a previous study using amino acid analysis showing an increase in total 3-NT in the brain after cerebral ischemia (Takizawa et al., 1999). N(G)-nitro-L-arginine methyl ester (L-NAME) administration antagonizes MRI changes and attenuates morphological abnormalities observed during reperfusion. This suggests that overproduction of NO, likely through peroxynitrite formation, may contribute to stroke-related cerebral injury. Under hyperglycemic conditions there was an early and concomitant increase in both superoxide and NO production, which can lead to peroxynitrite formation and therefore protein Tyr residue nitration, and thereby contributes to the damage in ischemic areas (Ste-Marie et al., 2001).

Demyelinating and inflammatory CNS diseases

The concentrations of ROS and RNS can increase dramatically under inflammation conditions. This can overwhelm the inherent antioxidant defenses within lesions, leading to cell death. Oligodendrocytes are more sensitive than glial cells and astrocytes to oxidative and nitrative stress due to their lower antioxidant capacity and their higher iron content (Smith et al., 1999). This results in preferential oligodendrocyte death and damage of myelin sheaths.

Results of studies in experimental models of allergic encephalomyelitis (EAE) and MS have provided strong evidence that an increase in peroxynitrite formation is associated with disease activity in the CNS (van der Veen et al., 1997; Cross et al., 1997).

When analyzed by immunohistochemical and *in situ* hybridization techniques, postmortem brain tissues of MS patients show an intense reactivity against iNOS mRNA and protein in reactive astrocytes, macrophages, and endothelial cells, and also against 3-NT, a putative footprint of peroxynitrite. Staining for 3-NT was not evident in chronic lesions in which only reactive astrocytes at the edge of the lesions were positive for iNOS (Liu et al., 2001). The close proximity of nitrotyrosine as well as iNOS immunoreactivity to the vessels may contribute to the blood-brain barrier (BBB) damage that is a cardinal feature of active MS lesions (De Groot et al., 1997; Oleszak et al., 1998).

Early reports focused on the possibility that increased NO production induced by proinflammatory cytokines IL-1 and IFN-γ is responsible for tissue injury. This possibility was based on the observations that RNS are toxic to oligodendrocytes, they stimulate axon demyelination, and also that NO inhibitors are protective against EAE development (Mitrovic et al., 1994; Dawson and Dawson, 1998; Smith et al., 1999; Zhao et al., 1996; Hooper et al., 1997; Okuda et al., 1998). However, recent findings have questioned the purely destructive role of NO. In particular, targeted deletion of the iNOS gene in mice enhanced their susceptibility to EAE and also specific iNOS inhibition was found to prolong the disease, supporting a putative role of NO as an immunosuppressive and anti-inflammatory molecule (Fenyk-Melody et al., 1998; Sahrbacher et al., 1998; O'Brien et al., 1999; Arnett et al., 2002). A potential explanation for the detrimental role of NO pathway activation is that NO per se is not responsible for myelin damage; rather, its downstream products such as PN could be the real damaging species (Willenborg et al., 1999). They are produced by the interaction between sensitized lymphocytes and activated macrophages/microglia as sources of proinflammatory cytokines and ROS, and by astrocytes that produce NO in response to cytokines. This hypothesis is consistent with data from EAE models, where there was widespread evidence of peroxynitrite formation in animals with active disease but not during disease recovery. Of great relevance in this regard are also the findings of Cross et al. (1998) who demonstrated the immunoreactivity for 3-NT in active lesions and the increase in nitrites and PN in the CSF of MS patients during a relapse. PN may lead not only to myelin disorganization but also to axonal damage and this can contribute to the permanent disability in MS patients (Touil et al., 2001). The increase in this marker of peroxynitrite production is not restricted to MS. Autopsy of CNS tissues from patients with AIDS-associated dementia complex showed an intense and widespread distribution of 3-NT, which was not evident in patients with HIV-associated encephalitis without dementia (Cross et al., 1997).

The CNS inflammatory response to neurotropic virus infection is also likely dependent on the activity of PN or its byproducts on the BBB (Hooper et al., 2001). Strategies aimed at antagonizing PN formation, such as the supplementation of ascorbic and uric acids, could therefore be potentially helpful, at least from a theoretical point of view, in maintaining BBB integrity, lessening CNS inflammation and destruction of target tissues in autoimmune and inflammatory diseases involving the CNS (Whiteman and Halliwell, 1996; Kean et al., 2000; Squadrito et al., 2000; Whiteman et al., 2002).

Table 1. Most relevant findings supporting nitrotyrosine formation in post-mortem brain tissues of patients affected by neurodegenerative diseases, stroke and Multiple Sclerosis

Disease (a) **Authors** **Findings** **Prevailing location** **Comments**
Parkinson's Disease
Affected areas Good et al., 1998 Increased 3-NT immunoreactivity Neurofilament proteins in Lewy body cores This observation provides a potential link between excitotoxicity and oxidative stress
Alzheimer's Disease
Affected areas Good et al., 1996 Increased 3-NT immunoreactivity Neurofibrillary tangles These findings link oxidative stress to NFT, and implicate NO expression and excitotoxicity in the pathogenesis of cell death in AD
Visual cortex Su et al., 1997 Increased 3-NT expression Terminal deoxynucleotidyl transferase neurons lacking evidence for tangle formation Neurons with DNA damage in the absence of tangle formation may degenerate by tangle-independent mechanisms and oxidative stress may contribute to such mechanisms
Parkinson's Disease **Dementia with Lewy bodies, Lewy body variant of Alzheimer's Disease** **Multiple system atrophy**
Affected brain areas Giasson et al., 2000 Widespread accumulation of nitrated alpha-synuclein Major filamentous building blocks of signature inclusions The selective and specific nitration of alpha-synuclein in these disorders provides evidence to directly link oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathies
Amyotrophic lateral sclerosis
Sporadic form: spinal cord Abe et al., 1995 Increased immunoreactivity for 3-NT Anterior horn motor neurons Contribution of peroxynitrite formation to motor neuron damage
Sporadic and familial form: lumbar and thoracic spinal cord Beal et al., 1997 Increased immunoreactivity for 3-NT and 3-nitro-4-hydroxyphenylacetic acid Motor neurons Peroxynitrite-mediated oxidative damage may play a role in the pathogenesis of both sporadic and familial ALS
Sporadic form: spinal cord Abe et al., 1997 Increased immunoreactivity for nNOS, eNOS, 3-NT Anterior horn motor neurons The nitration of protein-Tyr residues is upregulated in motor neurons of the spinal cord with selective increase of brain and endothelial NOS-like immunoreactivities
Sporadic form: cervical spinal cord Strong et al., 1998 3-NT immunoreactivity Neurofilament subunit protein (NFL) NFL is particularly susceptible to peroxynitrite-mediated damage
Ischemic Stroke
Autopsied brain ischemic stroke tissue Forster et al., 1999 iNOS and 3-NT immunoreactivity Neutrophils infiltrating the ischemic brain and in blood vessels within the ischemic territory The evidence of NO production and peroxynitration support the hypothesis that iNOS inhibitors may be useful in the treatment of stroke
Multiple Sclerosis
Autopsied brain tissues Severe form Bagasra et al., 1995 Detectable iNOS nitrotyrosine Monocyte/macrophages iNOS is expressed at significant levels in the brains of patients with MS and may contribute to the pathology associated with the disease
Autopsied brain tissues Severe form Van der Veen et al., 1997 Nitrotyrosine staining CD11b-positive cells (macrophages/microglia) PN is formed during progressive stages of disease activity
Autopsied brain tissues Cross et al., 1998 3-NT detected immunohistochemically MS sections displaying inflammation PN-mediated damage of CNS is associated with disease activity
Autopsied brain tissues Liu et al., 2001 Intense reactivity for iNOS staining for 3-NT (iNOS) reactive astrocytes in active lesions and edges of chronic lesions, Macrophages, inflammatory cells and endothelial cells in active lesions, 3-NT in acute but not chronic lesions, displayed a diffuse parenchymal membranous and perivascular pattern NO-mediated and peroxynitrite formation during the active phase and can concur in oligodendrocyte damage

(a): Brain areas from which the autopsied specimens were taken are specified, as reported by the Authors

Conclusion

The involvement of nitrative oxidation has been clearly demonstrated both in neurodegenerative and inflammatory disorders of the CNS. Nitrative oxidation is also

Table 2. Evidence for the production of nitrotyrosine in the CSF of patients affected by Parkinson's Disease, Alzheimer's Disease and Multiple Sclerosis

	Authors	Findings	Comments
Alzheimer's Disease			
	Teunissen et al., 2002	Increase in 3-NT, 8-OHdG, isoprostanes	Increase in nitrative and oxidative stress markers
Amyotrophic lateral sclerosis			
Sporadic	Tohgi et al., 1999c	Sevenfold increase of 3-NT and 3-NT/Tyr compared with controls	These data provide *in vivo* evidence for a possible role of peroxynitrites, a mediator of oxidative stress, and increased nitration of Tyr residues in the pathogenesis of sporadic ALS
Sporadic	Shaw & Williams, 2000	Increase of 3-NT and neurofilament light in CSF	These parameters, while not entirely disease-specific for ALS, may nevertheless be confirmatory markers of the disease and its progression
Not specified	Aoyama et al., 2000	Increased nitrated Mn-SOD	Slight increase also in Alzheimer's disease and Parkinson's disease
Multiple Sclerosis			
	Calabrese et al., 2002	iNOS was detected by Western blot Increase in NOS activity Greatly increased nitrotyrosine immunostaining of CSF proteins	These data strongly support a role for nitrosative stress in the pathogenesis of MS and indicate that therapeutic strategies focussed on decreasing production of NO by iNOS and/or scavenging peroxynitrite may be useful in alleviating the neurological impairments occurring during relapse

one of the mechanisms that contributes to the ischemic injury, particularly during the reperfusion phase.

The role played by RNS formation in the sequential pathophysiological mechanisms of brain damage in these pathologies remains to be established. The majority of studies comes from animal models and postmortem samples (Table 1). A few studies have been carried out *in vivo*, especially in the CSF, and longitudinal evaluation of the markers of peroxidation are lacking (Table 2).

Efforts should be made to verify the relevance of assessing peroxidation markers in relationship to patient outcome, particularly in light of a detailed identification of targeted and well-timed therapeutic strategies.

Acknowledgements

The authors express their gratitude to John A. Toomey for editing the English and Marisa M. Morson for technical assistance

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