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Oxidative stress and excitotoxicity: a therapeutic issue in multiple sclerosis?

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There is increasing evidence that multiple sclerosis (MS) is not only characterized by immune mediated inflammatory reactions but also by neurodegenerative processes. In neurodegenerative diseases, neuronal and axonal loss is mediated by oxidative stress and excitotoxicity which constitute a final common toxic pathway. Importantly, peroxynitrite is the key mediator of those two intertwined pathomechanisms. In MS, peroxynitrite is consistently associated with active lesions and produces highly toxic nitrating and oxidizing radical species that alter lipid, protein, DNA and mitochondrial structures and functions. During the remitting phase, peroxynitrite participates to neuron and oligodendrocyte damage in association with inflammatory processes. During the chronic phase, peroxynitrite contributes to self-perpetuating mechanisms responsible for disease progression. Neutralization of oxidative stress and excitotoxicity, and in particular of peroxynitrite derived free radicals, might represent a therapeutic approach to provide neuroprotection in MS. *Multiple Sclerosis* 2008; **14:** 22–34. http://msj.sagepub.com

Key words: endogenous neuroprotection; excitotoxicity; inosine; multiple sclerosis; oxidative stress; uric acid

Introduction

In most patients, multiple sclerosis (MS) is a biphasic disease initially characterized by remittent, acute neurological dysfunctions ultimately followed by a progressive increase in disability. Clinical, radiological and pathological observations support the occurrence of different pathomechanisms during the relapsing-remitting (RR) and the secondary progressive (SP) stages, the RR phase being associated with transient, immune mediated inflammatory reactions and the SP phase with steady, neurodegenerative processes. A great deal of information has emerged recently concerning the molecular pathomechanisms involved in neurodegeneration. The role of inflammatory processes, particularly of microglial cell activation, has received increasing attention as the primary reaction leading to oxidative stress and excitotoxicity [1]. These interacting processes converge to play a pivotal role in neuronal death [2] and, importantly, the central role of peroxynitrite as the potent final effector molecule has been demonstrated [3]. In experimental allergic encephalomyelitis (EAE) [4] and in MS, microglial activation is an early event inducing the inflammatory response [5] subsequently leading to oxidative stress and excitotoxicity. The respective roles of overlapping inflammatory and neurodegenerative processes in MS pathogenesis vary over the course of the disease. During the RR phase, inflammation predominates associating microglia activation and the presence of important cellular infiltrates responsible for neural tissue damage. Importantly, oxidative stress and excitotoxicity can already be demonstrated. The SP phase is characterized by the presence of activated microglia without a prominent cellular component. Oxidative stress and excitotoxicity seem to prevail and lead to an auto-toxic loop actively participating to neurodegeneration. Inactivation of oxidative stress, excitotoxicity and in particular of peroxynitrite derived toxic radicals, may thus represent a promising approach to delay neuronal and axonal loss, the most important factor in determining the final disability in MS.

Oxidative toxicity in multiple sclerosis

Excessive production of nitric oxide (•NO) is the driving force for peroxynitrite formation. Nitric oxide is an important biological messenger and plays a pivotal role in neurophysiology. However

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•NO can be neurotoxic as well as neuroprotective and it is the amount of •NO and the biological environment that determine its physiologic or pathophysiologic action. In the central nervous system (CNS), three nitric oxide synthases (NOS) isoforms can generate •NO. Excessive production of •NO by activation of constitutive neuronal NOS (nNOS) is observed during mitochondrial respiratory chain dysfunction. Inducible NOS (iNOS) is located predominantly in macrophages and microglia and generate •NO in high levels and for extended periods during inflammatory reactions. Enhanced expression of the third isoform, constitutive endothelial NOS (eNOS) and subsequent local production of •NO is associated with blood brain barrier (BBB) dysfunction. A fourth isoform has been recently identified, the mitochondrial NOS (mtNOS), producing reactive nitrogen species (RNS) via the respiratory chain.

Peroxynitrite (oxoperoxonitrate [−1]: $ONOO^-$) is generated *in vivo* by the combination of two ubiquitous free radicals: superoxide ($O_2•^-$) and •NO [6]. Peroxynitrite is rapidly protonated to peroxynitrous acid (hydrogen oxoperoxonitrate) that homolyzes to form nitrogen dioxide ($•NO_2$) and the hydroxyl radical (•OH). For the most part, they recombine to form nitrate, a non-toxic product, but 30% lead to the formation of the highly toxic nitrite compound. In our biological environment, given the high concentration of the bicarbonate/carbon dioxide (CO_2) pair, peroxynitrite reacts rapidly with CO_2 to form $•NO_2$ and carbonate radicals ($CO_3•^-$), two strong oxidants [7].

Most of peroxynitrite pathomechanisms responsible for the protein and enzyme nitration are mediated via derived secondary oxidizing species. Highly toxic, peroxynitrite can nitrate and oxidize lipids, proteins, DNA and carbohydrates. In particular, peroxynitrite nitrates tyrosine residues, resulting in nitrotyrosine (NT) that can be identified immunohistochemically as a specific marker of peroxynitrite toxicity.

The presence of peroxynitrite in MS lesions as demonstrated by NT reactivity has been extensively investigated [8–10]. In acute lesions with intense perivascular and parenchymal infiltration, most NT-positive cells are mononuclear inflammatory cells. In chronic active lesions showing perivascular infiltrates and ongoing demyelination, NT-positive cells are predominantly lipid-laden macrophages, perivascular cells and ependymal cells from periventricular lesions. A prominent expression was noted in plaques with high inflammation. In contrast, in chronic silent lesions lacking an inflammatory component, NT-positive cells are absent [11]. In EAE, peroxynitrite is formed very early, correlates with disease activity and cannot be detected during remissions and chronic, silent stages [12]. Both in MS and EAE, there is thus a clear correlation between inflammatory processes and peroxynitrite formation.

The predominant pathogenic role of peroxynitrite in EAE has been demonstrated after injection of a peroxynitrite catalyst specifically neutralizing peroxinitrite activity without affecting •NO mediated mechanisms [13]. Incidence and severity of clinical signs, inflammatory reactions, as well as NT immunoreactivity were markedly reduced. The prominent role of peroxynitrite is also reflected by the observation that mature oligodendrocytes are relatively resistant to direct •NO mediated toxicity but exquisitely sensitive to peroxynitrite-mediated injury [14].

In vivo damages of myelin and axons induced by peroxynitrite have been investigated in the rat [15]. Injection into the corpus callosum of a spontaneous donor of peroxynitrite produced vacuolization and destruction of myelin, similar to MS lesions. Amyloid precursor protein staining, a marker of axonal damage, was closely associated with demyelination and histopathology showed lesions characteristic of acute axonal lesions.

Due to their low antioxidant defences, neuronal cells are particularly vulnerable to oxidative toxicity. In EAE, peroxynitrite nitration of mitochondrial proteins is an early event, preceding infiltration of inflammatory cells and leading to several mitochondrial dysfunctions: respiratory chain depression, ATP synthesis reduction, membrane potential attenuation, imbalance in calcium homeostasis, reactive oxygen species (ROS) production, opening of permeability transition pores and DNA single strand breaks. Those peroxynitrite-induced mitochondrial impairments prime apoptosis of neurons and oligodendrocytes as well as axonal degeneration [16]. Tyrosine nitration by peroxynitrite is associated with inactivation of protein functions, especially of neurofilament-L proteins expressed in motor neurons and essential for their survival [17] as well as mitochondrial proteins, among which cytochrome c, Mn superoxide dismutase (SOD) and ATPase [18]. In MS, oxidative damage to mitochondrial DNA and mitochondrial enzymes have been observed in active chronic lesions [19]. Interestingly, in the pattern III subset of MS, mitochondrial respiratory dysfunction caused by oxidative stress results in pathological lesions similar to those observed in histotoxic hypoxia [20]. Mitochondrial impairment definitely plays an important role in MS progression [21,22]. Lastly, peroxynitrite can transform nerve growth factor into an apoptotic factor for motoneurons at physiological concentration [23].

Astrocytes contain high concentrations of antioxidant enzymes and play a role in antioxidative processes [24]. However, inflammation induces iNOS

expression in astrocytes leading to production of •NO that permeates astrocyte cell membrane and reacts with O_2•$^-$ to form peroxynitrite. The latter appears more deleterious to the neighbouring neurons than to the astrocytes themselves, but in response to extracellular peroxynitrite, astrocytes may become cytotoxic to motoneurons via peroxynitrite-dependent mechanisms [25].

Like neurons, oligodendrocytes have low levels of antioxidant enzymes. Combined with a high iron content, this put them at increased risk for oxidative stress [26]. Peroxynitrite was found to mediate microglial toxicity to oligodendrocytes [27]. Importantly, several observations have demonstrated a maturation-dependent susceptibility of oligodendrocytes to free radical attacks [28,29]. Oxidative toxicity could thus play a role not only in myelin destruction, but also in counteracting remyelination.

A strong expression of eNOS, a potential source of peroxynitrite, has been demonstrated in the intraparenchymal vascular endothelial cells of capillaries and venules of MS brains [30]. During their homing into CNS, overexpression of iNOS and •NO occurs in activated monocytes [31], and this may contribute to peroxynitrite formation inside endothelial cells and consequent damage of the BBB. Peroxynitrite also causes tight junction damage by altering zona occludens-1, f-actin and tight junction associated proteins [32] as well as other junctional protein expression [33]. It thus contributes to BBB dysfunction by disrupting both transcellular and paracellular paths [34].

Lastly, free iron certainly plays a role in the pathogenesis of MS by catalyzing Fenton reactions that lead to oxidative damage. However, several therapeutic interventions targeting iron metabolism in EAE and MS patients remained inconclusive so far [35].

Excitotoxicity in multiple sclerosis

Preliminary observation demonstrated increased glutamate and aspartate CSF levels in MS patients during the acute phase strongly suggesting a role for glutamate mediated toxicity (excitotoxicity) [36]. Increased glutamate and aspartate CSF levels have been confirmed in patients with RR MS during relapses and even during a clinically stable phase when there was MRI evidence of active lesions [37]. Using high field MR spectroscopy, significantly higher glutamate levels compared to controls were found not only in active and chronic lesions, but also in normal appearing white matter in MS patients [38].

Both neurons and mature oligodendrocytes express amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)/kainate as well as N-methyl-D-aspartate (NMDA) receptors and are exquisitely vulnerable to glutamate toxicity while astrocytes are more resistant [39].

Excessive activation of glutamate ionotropic receptors causes dysregulation of calcium homeostasis [40] and triggers calcium influx into surrounding cells. The initial calcium entry in neuronal or oligodendroglial cells is not toxic per se, but the further uptake of calcium in the mitochondrion entails mitochondrial calcium overload, subsequent O_2•$^-$ production, calcium dependant •NO generation and peroxynitrite formation [41]. The combined action of oxidative stress and calcium overload results in opening of the mitochondrial permeability transition pore [42], leading to cell death via destruction of molecules necessary for cell survival (necrosis) or initiation of a programmed cell suicide (apoptosis). AMPA/kainate receptors are particularly robust in immature oligodendrocytes, making them exquisitely sensitive to excitotoxicity [43]. In addition, activation of kainate, but not AMPA receptors, sensitizes oligodendrocytes to complement toxicity [44]. Myelin can be damaged by glutamate but axons are resistant [45]. Like oxidative stress, excitotoxicity may contribute to BBB dysfunction via activation of endothelial cell ionotropic glutamate receptors [46].

A dysregulation of excitatory amino acids in EAE and a potential beneficial effect of their antagonists have long been suggested and administration of NMDA blockers was found effective in reducing neurological deficits [47]. It has been also suggested that alterations in glutamate homeostasis might be excitotoxic to oligodendrocytes and that the lesions caused by overactivation of ionotropic glutamate receptors resemble those observed in MS [48].

Experiments with an AMPA/kainate antagonist (NBQX) demonstrated a reduced axonal and oligodendroglial damage [49,50]. The absence of beneficial effects on inflammatory reactions and lymphocyte proliferation indicates that the benefit was not mediated via the immune system. Other experiments in adoptive transfer EAE and in chronic-relapsing EAE confirmed this specific role of various AMPA/kainate antagonists [51]. In MS, excitotoxicity produced by extracellular glutamate overload may result from different pathomechanisms. First, mitochondrial respiratory chain impairment is a consistent feature in neurons exposed to peroxynitrite and is associated with glutamate ionotropic receptor activation. Second, peroxynitrite inhibits glutamate transporters in a non-specific way leading to increased extracellular glutamate levels [52]. Third, it has been recently demonstrated that TNF-alpha produced by activated microglia stimulates glutamate release by up-regulating glutaminase [53].

Immunochemistry in MS and control white matter [54] consistently indicate axonal damage in areas showing altered glutamate homeostatis, colocalized with strongly positive glutamate positive cells. Importantly, a major glial glutamate transporter (GLT-1) as well as two enzymes, glutamate dihydrogenase (GDH) and glutamine synthase (GS), are lost from oligodendrocytes surrounding active lesions even long after inflammatory reactions have subsided. It is also noteworthy that oligodendrocytes play an essential role in the removal of extracellular glutamate [55].

Lastly, a significant decrease in glutamate-induced inhibition of the proliferation of T lymphocytes from MS patients has been demonstrated, which could prolong the inflammatory response and favour a progressive evolution [56].

Neurodegeneration and multiple sclerosis

In recent years, experimental and clinical observations have provided new insights about pathomechanisms responsible for the progressive evolution of neurodegenerative disorders. Two important concepts have emerged: first, both oxidative stress and excitotoxicity are the common final pathomechanisms (but not the primary cause) of neuronal injury due to diseases with diverse pathophysiologic processes; second, peroxynitrite is the common driving force for those two, closely intertwined, pathomechanisms. Importantly, inflammation can lead to oxidative stress and conversely. In MS, oxidative toxicity and excitotoxicity are narrowly linked. It has been recently demonstrated indeed, that iNOS and cyclooxygenase-2 (a protein that accelerates glutamate-mediated apoptosis) are coexpressed in chronic active lesions, particularly in microglia/macrophages [57].

Neurons and oligodendrocytes (particularly their progenitors) are exquisitely sensitive to oxidative stress and excitotoxicity. Peroxynitrite is capital to initiate neuron and oligodendrocyte necrosis or apoptosis via its derived nitrating and oxidizing free radicals. In addition to their direct toxicity resulting from the nitration and oxidation of proteins and lipids, free radicals cause mitochondrial dysfunction leading to neural cell degeneration via intracellular Ca overload.

The precise pathomechanims by which axons degenerate remain to be elucidated. During the acute phase, demyelination predominates and makes axons vulnerable to inflammatory mediators as well as to cellular and antibody-mediated damages partly reversible. Interestingly, MS patient CSF-derived scFv Ab bind to axons in acute but not in chronic lesions [58]. During the chronic stage, demyelinated axons become susceptible to hypoxic/ischemic damage resulting from oxidative toxicity and excitotoxicity. It has come to be accepted that co-expression of iNOS and NT closely localized to damaged axons implies that they have been exposed to toxic effect of peroxynitrite [59]. Peroxynitrite avidly interacts with mitochondrial components, leading to mitochondrial electron transport chain dysfunction and to reduced capacity of ATP production. Microarray analyses of brain tissue from MS patients have recently confirmed a significant reduction in gene products specific for the electron transport chain, particularly in motor neurons [60]. Moreover, immunochemistry has demonstrated a reduction in inhibitory neurotransmission at the synaptic levels. It is thus postulated that the occurrence of an increased firing of demyelinated axons, consequent to the reduction in inhibitory transmission, and a simultaneous decrease in ATP production may cause axonal degeneration via an imbalance between energy demand and supply. Increased numbers of mitochondria in demyelinated axons appears related to the enhanced energy demand and is considered as an early sign of ensuing axonal degeneration [61].

It is of interest that numerous observations in neurodegenerative diseases led to the hypothesis that peroxynitrite plays a central role in sustaining as well as in initiating an 'auto-toxic loop' responsible for their progressive pathology [2,18,62–64]. According to this self-perpetuating process, initial inflammatory reactions lead to the formation of peroxynitrite that induces glutamate overload via inhibition of glutamate transporters and/or via glutamate receptor activation. The resultant glutamate overload causes mitochondrial energy metabolism impairment. Electron transfer uncoupling and decreased capacity to reduce molecular oxygen, entail $O_2\bullet^-$ production. Concurrently, mitochondrial Ca2+ overload generates •NO that reacts with $O_2\bullet^-$ leading to secondary production of peroxynitrite which, in turn, can reinitiate the cycle (Figure 1). Given that peroxynitrite is consistently associated with active chronic lesions in MS, the proposed feedforward pathological cycle proposed in other neurodegenerative processes might contribute, at least in part, to disease progression.

In neurodegenerative diseases, the role of microglial activation as the primary event leading to peroxynitrite production has been recently demonstrated, but the specific causes of this initial inflammatory process have not been clearly identified. In Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis, genetic factors seem responsible for the accumulation of intra and extracellular aggregates [65] that interact with cellular membranes causing cell death, subsequent microglial activation and oxidative stress [66]. In MS, inflammation might be initiated by autoreactive

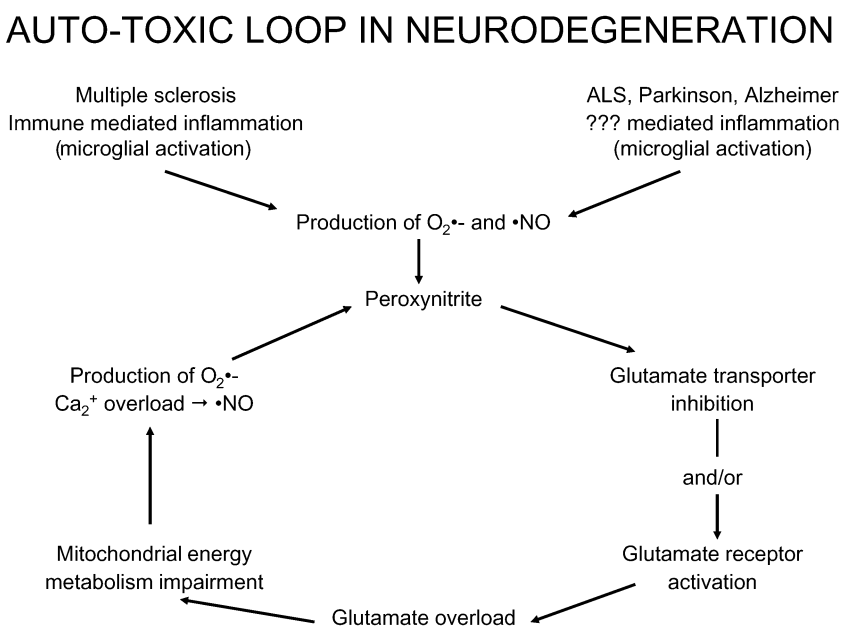

Figure 1 Both in MS and in neurodegenerative diseases, inflammation initiates microglial activation. In MS, inflammation is immune-mediated but in neurodegenerative diseases the primary causes of inflammation remain speculative. Activated microglia produce O_2•$^-$ and •NO free radicals that combine to induce primary synthesis of peroxynitrite. Peroxynitrite inhibits glutamate transporters and/or activates glutamate receptors causing glutamate overload. The resulting excitotoxicity entails mitochondrial dysfunction, calcium overload and subsequent production of •NO and O_2•$^-$. Their recombination leads to the secondary synthesis of peroxynitrite that reinitiates the cycle.

T cells generated in the systemic compartment that gain access to the CNS (systemic hypothesis) or by a primary dysfunction or death of oligodendrocytes or neurons causing microglial activation (neural hypothesis) [67].

Incidentally, these two hypotheses might not be mutually exclusive. It is of note that in both MS and neurodegenerative diseases, oxidative stress and excitotoxicity are a common final pathomechanism and not a common priming event causing neurodegeneration.

Peroxynitrite toxicity inactivation

Oxidative stress, and peroxynitrite in particular, plays a capital role not only in the pathogenesis of neurodegeneration but also of diverse pathological states (atherosclerosis, preeclampsia, diabetic complications and others). The identification of peroxynitrite as the potent final effector molecule has fostered a wealth of research to neutralize its toxicity. Extensive reviews of pharmacological strategies available to modulate iNOS and its mediators [68] as well as of drugs modulating the biological effects of peroxynitrite [69] have been recently published. This survey will be limited to the most promising approaches targeting peroxynitrite and its by-products (Table 1).

Synthetic compounds

Peroxynitrite catalysts are water-soluble manganese and iron porphyrins that decompose peroxynitrite directly to harmless nitrate without producing toxic nitrating species. They outcompete the reaction of peroxynitrite with CO_2 and scavenge the highly toxic peroxynitrite derived CO_3•$^-$ radical [70]. In EAE experiments, inflammatory processes were considerably reduced as well as NT immunoreactivity but no effect on axonal necrosis was observed. Peroxynitrite catalysts did not delay disease onset and there was only a trend toward reduction of clinical severity [13].

Nitroxides are stable free radicals extensively used as spin labels and MRI contrast agents since several decades for biophysical metabolic studies (oxygen tissue concentration). They protect against peroxynitrite, •NO, and CO_3•$^-$ toxicities. They also compete with peroxynitrite for CO_2, diverting peroxynitrite/CO_2 reactivity from the deleterious protein nitration to the harmless protein nitrosation. Accordingly, nitroxides sharply inhibit protein-tyrosine nitration by 70–90% [71]. A nitroxide, Tempol, has proved very effective in several experimental models of diseases mediated by oxidative stress, but has not been tested in EAE or in preliminary clinical trials so far.

Table 1 Experimental therapeutic approaches of oxidative stress and excitotoxicity

Mechanisms of action	Comments
Oxidative stress	
•NO, iNOS inhibitors	Blocking iNOS may prevent the physiological protective role of •NO [116]
Peroxynitrite catalysts	Moderate efficacy in EAE [13]
Nitroxides	Effective in other experimental models [121], not tested in EAE
Nitrones	Efficacy in stroke not confirmed [74], not tested in EAE
Free radical scavengers	
Ascorbic acid	More effective in combination with uric acid [79]
Uric acid	The most effective radical scavenger in EAE [82], well tolerated in MS [92]
Glutathione inducers	
α-tocopherol	Not tested in EAE, γ-tocopherol appears more appropriate [94]
N-Acetyl-L-cysteine	Marked efficacy in EAE, NAC-amide permeates the BBB [97]
Thymoquinone	Has both radical scavenging and anti-inflammatory properties [99]
Biliverdin reductase	Effective in EAE without increasing bilirubin serum levels [103]
Excitotoxicity	
NMDA antagonists	
Memantine	Abrogates clinical signs but not inflammation in EAE [47]
Riluzole	Reduces spinal cord atrophy progression in MS [122]
Antagonists of Ca overload	
Na channel blockers	
Flecainide	Well tolerated in patients with long QT-syndrome, no trial in MS [123]
Lamotrigine	Effective in EAE [112], clinical trial in MS in progress [124]
Phenithoine	Effective in EAE [113], clinical trial in MS in progress [124]
K channel blocker	
Tram-34	Ameliorates relapsing EAE [114], no trial in MS
Transcriptional therapy	
Caspase inhibitors	
Trichostatin A	Upregulates antioxidative, anti-excitotoxic, trophic factor activity [125]
PARP-1 inhibitors	
Phenanthridinone	
Benzamide	Effective in EAE, no trial in MS [126]
Benzoic acid	
Bcl-2 antiapoptotic family	
Bcl-XL protein fusion	Protects retinal ganglion cells in EAE [127]
Pramipexole	Effective in experimental Parkinson [128]
Erythropoietine	Effective in EAE, carbamylated-EPO does not increase erythrocyte production [110]

Anti-oxidative and anti-excitotoxic molecules most frequently investigated in experimental models, notably EAE. Most of those molecules have several mechanisms of action making their classification difficult.

Nitrones are used in biochemistry to trap and stabilize free radicals for their identification. Like nitroxides they inhibit CO_3•$^-$ and •NO_2 oxidation/nitration [72]. They exert neuroprotective effects in several models of neurodegenerative diseases. One of them (NXY-059) was found effective to reduce disability after acute ischemic stroke in a preliminary study [73] but larger trials did not show a statistically significant benefit [74].

Natural antioxidant defences

In aerobic life, given that the catabolism of molecular oxygen produces ROS, natural antioxidant defences have been progressively integrated in biological systems. Endogenous protection against oxidative damages includes enzymes (SOD, catalase, peroxydases) as well as low molecular weight antioxidants such as alpha-tocopherol, GSH, cysteine, ascorbic acid, bilirubin and uric acid (UA).

Most importantly, it has been demonstrated in EAE that •NO and O_2•$^-$ alone are not sufficient for the peroxidation of myelin and that they are required simultaneously to exert an oxidative toxicity via peroxynitrite generation [75]. Experiments using antagonists (SOD, catalase and peroxydase) of precursors of peroxynitrite (•NO, O_2•$^-$) as well as pharmacological agents modulating •NO or iNOS yielded contrasting results [76].

In hominoids, roughly 20 million years ago, improvement in antioxidant defences, in particular in free radical scavengers, was achieved by inactivation of the uric acid oxidase (uricase) gene [77]. The resulting inability to change UA in allantoïne has increased plasma UA levels by 10 times in humans compared with other mammals. Simultaneously, the ability to synthesize ascorbic acid was lost. The replacement of ascorbic acid with UA as the major natural antioxidant is considered as an evolutionary advantage.

In humans, UA is the terminal degradation product of adenine and guanine-based metabolism and was long regarded as waste products. However, since the early 1990s, the specific antioxidant properties of UA have received increasing attention. Uric acid comprises 30–65% of the peroxyl radical-scavenging capacity of the plasma. On an equimolar basis, UA is 10 times as effective as ascorbate to scavenge singlet oxygen, hydrogenperoxide, •OH, O_2•$^-$, •NO, •NO_2 and CO_3•$^-$ [78]. Concentrations of UA are lower in the CNS than in other organs, and brain protein homogenates are more sensitive to peroxynitrite toxicity than heart protein homogenates [79]. Interestingly, it has been recently shown that ischemic preconditioning protection is partly mediated by natural antioxidant mobilisation, especially UA [80].

The efficiency of UA as neuroprotectant is definitely related to its ability to scavenge peroxynitrite-derived radicals before they can react with their targeted biological molecule. Indeed, the reaction of peroxynitrite with CO_2 to form nitrosoperoxycarbonate, which rapidly homolyzes to form NO_2•$^-$ and CO_3•$^-$, is 1000 times faster than the corresponding reaction with UA [81]. It has been demonstrated that •NO_2 and CO_3•$^-$ free radicals play a crucial role in peroxynitrite-mediated toxicity. Nitrogen dioxide preferentially reacts in environments such as cell membranes and hydrophobic protein domains, while CO_3•$^-$ can initiate lipid peroxidation, produce nitrated lipids and oxidize amino acids. Relevantly, UA concentrations around those present in human plasma inhibit completely tyrosine nitration *in vitro* [7].

Radical intermediates can be generated during the reactions of UA with peroxynitrite, notably the urate radical. The latter however is rapidly converted back to urate by ascorbate. There is thus a co-operative interaction between the two most important natural antioxidants, UA and ascorbate. Experimentally, the antioxidant efficacy of UA in combination with ascorbate was found three times more important than that of UA alone. Ascorbate improves the ability of UA to act as an antioxidant by inhibiting radical intermediates generated by the interaction of UA with peroxynitrite [79].

Hooper and his group compared the protective effect of UA in acute EAE with that of a •NO scavenger (c-PTIO) and an iNOS inhibitor (D609). Only UA completely protected mice from EAE [82] and had a therapeutic effect when administered in animals with pre-existing disease. They also reported that serum UA levels were lower in patients with MS [83]. Reduced serum UA levels in MS are likely secondary to its peroxynitrite derived free radical scavenging activity rather than to a primary deficiency [84]. In addition, a survey of 20 212 505 Medicare and Medicaid records revealed that the incidence of MS in persons with chronic hyperuricemia (gout) was about 10 times lower than expected [83].

The BBB is poorly permeable to UA but it is assumed that its compromised integrity in EAE facilitates the entrance of UA into the brain and that monocytes invading inflammatory lesions serve as a short-term reservoir of UA [85]. On the other hand, the BBB dysfunction associated with acute EAE is restored during UA administration [86]. The proposed mechanism is an inhibition of the production of TNF alpha by UA in neurovascular endothelial cells, a cytokine that upregulates ICAM-1 expression [87].

The antioxidant activity of UA was compared *in vitro* with that of ascorbic acid, cysteine and GSH, and UA proved to be the most effective scavenger [88]. Uric acid had no effect on antigen presentation and recognition as well as on T cell priming and expansion [89] and did not affect the production of •NO [86].

In humans, oral administration of UA does not increase serum UA levels because of its destruction by uricase produced by microbial flora. Inosine and inosinic acid, two precursors of UA, proved to be able to increase serum UA in mice and to inhibit EAE development [90]. It is noteworthy that neither inosine nor inosinic acid reacted with peroxynitrite *in vitro* and that an increased concentration of UA, but not of inosine, was observed in spinal cord tissues. The protective effect obtained after oral administration of inosine appears thus mediated by its metabolite UA. Interestingly, oral administration of inosine in MS patients entails a marked increase in cerebrospinal fluid UA levels [91]. In preliminary clinical trials in RR MS, inosine administration appears safe [92] and a trend towards a reduction in progression rate was reported [93].

There is less information about the potential protective activity of other low molecular weight radical scavengers. Alpha-tocopherol and GSH have not been investigated in EAE. It is noteworthy that the weak alpha-tocopherol antioxidative activity is definitely potentiated by gamma-tocopherol which is required to effectively remove the peroxynitrite-derived nitrating species. Our current rationale of vitamin E supplementation with primarily alpha-tocopherol as an antioxidant should thus be reconsidered [94]. Oral administration of N-acetyl-L-cysteine (NAC), a GSH inducer, substantially inhibited the development of EAE [95] in mice, and a recent publication demonstrates a reduction in the cellular infiltrate in the CNS [96]. However, NAC administration did not delay the onset of clinical signs likely because of the low ability of NAC to cross the BBB. A newly designed amide form of NAC (NAC-amide) crosses the BBB [97] and has proved able to protect

animals completely against EAE induction. Interestingly, NAC reduces by 50% the expression of genes regulating pathological pathways triggered by oxidative toxicity in EAE [98]. Another GSH inducer, thymoquinone, has been recently reported to prevent and to ameliorate EAE as well as to inhibit NF-kB activation and the consequent inflammatory gene expression [99].

Like UA, bilirubin has long been regarded as an end waste product of heme catabolism. Heme oxygenase cleaves the heme protein to form biliverdin that in turn is rapidly reduced to bilirubin by biliverdin reductase (BVR). Given its high rate reduction, biliverdin does not seem to have specific functions but bilirubin [100] and BVR [101] exert potent antioxidative activities. Bilirubin was found effective in EAE [102] and it has been recently demonstrated that BVR suppresses pathological and clinical signs of EAE more efficiently than other natural antioxidants [103]. Biliverdin reductase was not compared with UA however. Interestingly, this beneficial effect is obtained with doses that do not increase bilirubin serum concentrations which could be toxic.

Discussion

A considerable amount has been learned recently about pathomechanisms involved in neurodegeneration. A compelling body of evidence has emerged that in MS, immune-mediated inflammatory processes are the proximate cause initiating oxidative stress, excitotoxicity and the mitochondrial injury cascade ultimately leading to neuronal, axonal and oligodendroglial loss. Moreover, at a certain stage of disease evolution, oxidative stress and excitotoxicity initiate self-activated pathways that do not require any further immune-mediated inflammatory processes.

The incidence of the dichotomy between inflammatory-mediated immune pathomechanisms and neurodegenerative processes on the therapeutic benefit of immunosuppressive drugs has been clearly demonstrated in EAE [104]. Interestingly, after a drastic immunosuppression with alemtuzumab in early RR MS patients (mean disease duration: 2.7 years), not only relapses were suppressed, but the EDSS score was reduced by 1.4 points over 2 years [105]. In contrast, the same treatment administered later in the course of the disease (SP MS) had no effect on progression [106]. A very early suppression of the inflammatory environment reduces the concomitant production of microglial toxic factors, in particular the primary synthesis of peroxynitrite that initiates the pathological cascade leading to progressive CNS tissue destruction. During the progressive phase, anti-inflammatory drugs are less effective on disability progression suggesting that the pathogenesis of MS lesions is different between the RR and the SP stages. A first explanation might be the sequestration of inflammation within the intrameningeal ectopic lymphoid follicles recently described in the brains of patients with SP [107]. This raises indeed the question whether immunosuppressants gain access to those sequestered inflammatory foci after systemic administration. Another explanation might be that acute inflammation no longer predominates at that stages and that prominent self-perpetuating pathomechanisms lead to intracellular Ca overload, mitochondrial impairment, $O2\bullet^{-}$ and •NO production and subsequent peroxynitrite synthesis without the support of inflammation.

Detailed mechanisms of peroxynitrite toxicities are not fully clarified but caspase and poly(ADP-ribose) polymerase-1 (PARP-1) activation likely determines the type of cell death: necrosis versus apoptosis. Accordingly, caspase and PARP-1 inhibitors were found to protect against peroxynitrite toxicity [108]. Another interesting concept now emerging is that peroxynitrite-derived ROS and RNS triggers mitogen-activated protein kinase which activates certain transcription factors, such as the nuclear factor-kappaB (NF-kB). The latter upregulates the expression of genes responsible for the activation of crucial pathomechanisms involved in MS, like apoptosis, inflammation and demyelination [97,109]. Lastly, given the exquisite sensitivity of oligodendrocyte progenitors to oxidative toxicity, peroxynitrite also counteracts remyelination.

Mitochondrial dysfunction plays a capital role in neurodegeneration leading to apoptosis, the terminal event. A small number of anti-apoptotic compounds are in development for the treatment of neurodegenerative diseases. So far, animal experiments have provided evidence for prevention of cell death, but little for functional benefit. Erythropoietin (EPO) acts in a signalling cascade which converges on effectors of the Bcl-protein family to protect neurons from apoptosis. Effective in EAE, carbamylated-EPO, that does not affect erythrocytes production, has been proposed for the treatment of MS patients [110].

Calcium overload represents a common pathomechanism of cell injury and is mediated through the activation of several Ca-dependent pathways including glutamate receptors. AMPA/kainate receptors appear primarily involved and AMPA blockers were found effective in EAE [51]. However, altering physiological glutamate signals with glutamate receptor blockers could lead to unwanted adverse side effects, potentially avoided with glutaminase inhibitor administration [59]. Most of intracellular Ca accumulation pathways are Na or K channel dependent. Na channel antagonists appear

therefore an attractive approach and were found effective in EAE [111–113], as well as K channel antagonists [114]. Yet, it is to be expected that therapeutic approaches based on the reduction of only one or even a few of the Ca-dependent systems acting far downstream in the mitochondrial injury cascade after Ca has been released, will not be sufficient to provide a clinically effective neuroprotection [115]. Treatment strategies targeting more proximal upstream events leading to Ca homeostasis dysregulation might be more relevant. It has been consistently demonstrated that peroxynitrite damages enzymatic functions leading to intracellular Ca overload.

Antagonists of peroxynitrite or of its precursors appear interesting candidates. Peroxynitrite catalysts and nitroxides were found effective in experimental models, but they have not been tested in clinical trials so far. Blocking peroxynitrite synthesis with •NO and/or $O_2•^-$ antagonists yielded only modest and contrasting benefits in EAE [75]. The timing of administration clearly determines the effects: early administration reduces while delayed administration aggravates disease evolution [116]. The likely reason is that •NO is a pathogenic factor during the induction phase but plays an inhibitory role during the progressive phase. The exacerbation of EAE in mice lacking the NOS2 gene illustrates the protective role of iNOS and •NO [117]. Peroxynitrite catalysts and nitroxides were found effective in experimental models, but they have not been tested in clinical trials so far.

Natural autoprotective mechanisms are certainly activated in MS as demonstrated by the upregulation of gene expression in several pathways related to oxidative stress and ischemic preconditioning [118]. However they only partially counteract lesion formation and do not prevent disease progression. Among natural antioxidant defences, UA appears the most efficient in EAE. Its efficacy is clearly related to its ability to scavenge peroxynitrite derived radicals before they can react with their targeted biological molecules, in particular with mitochondrial enzymes, and consequently to prevent the mitochondrial injury cascade. It is worthy of note that UA actively participates to endogenous neuroprotection, particularly to the development of ischemic tolerance [80].

Conclusions

Our current understanding of successive pathomechanisms involved from the relapsing to the progressive stage in MS suggests that the most logical therapeutic strategy would be to eradicate acute inflammatory processes and to reduce concomitant neuronal and axonal damage as soon as clinical and/or radiological signs of disease activity are observed. So far, only potent immunosuppressants are able to achieve such an outcome but unfortunately, none of them has an acceptable tolerance for this indication. Well tolerated, potent immunosuppressants are thus eagerly awaited. The next step would necessarily be a combined therapy associating an anti-inflammatory drug and a neuroprotective agent. Currently available immunomodulators might be able to contain the moderate, residual inflammatory processes more effectively than acute inflammation. However, they do not protect neurons from oxidative stress and excitotoxicity and neuroprotection could be achieved only with agents interfering with one or several mechanisms leading to the auto-toxic loop responsible for neurodegeneration. Targeting peroxynitrite-induced cytotoxic pathways seems thus a promising strategy. Importantly, oxidative stress activates microglia that lead to the production of pro-inflammatory cytokines. Antioxydants provide thus an additional, indirect neuroprotection through suppression of glia-mediated inflammation [119]. It is worthy of note that combined blockade of inflammation and neuroprotection against excitotoxicity in EAE resulted in clinical improvement and repair of the CNS [120]. Based on our current understanding of the disease pathogenesis, combining early several therapeutic approaches appears a prerequisite in MS.

Abbreviations

•NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial NOS
iNOS	inducible NOS
nNOS	neuronal NOS
$O_2•^-$	superoxide
$•NO_2$	nitrogen dioxide
•OH	hydroxyl radical
CO_2	carbon dioxide
$CO_3•^-$	carbonate radical
NT	nitrotyrosine
EAE	experimental allergic encephalomyelitis
SOD	superoxide dismutase
BBB	blood brain barrier
ROS	reactive oxygen species
RNS	reactive nitrogen species
NF-kB	nuclear factor kappa B
NMDA	N-methyl-D-aspartate
AMPA	amino-3-hydroxy-5-methyl-4-isoxazolepropionate
GLT	glutamate transporter
GDH	glutamate dehydrogenase

GS glutamine synthase
GSH glutathione
UA uric acid
NAC N-acetyl-L-cysteine
BVR biliverdin reductase

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