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REVIEW

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The role of oxidative stress in the pathogenesis of multiple sclerosis: The need for effective antioxidant therapy

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Abstract Accumulating data indicate that oxidative stress (OS) plays a major role in the pathogenesis of multiple sclerosis (MS). Reactive oxygen species (ROS), leading to OS, generated in excess primarily by macrophages, have been implicated as mediators of demyelination and axonal damage in both MS and experimental autoimmune encephalomyelitis (EAE), its animal model. ROS cause damage to cardinal cellular components such as lipids, proteins and nucleic acids (e. g., RNA, DNA), resulting in cell death by necrosis or apoptosis. In addition, weakened cellular antioxidant defense systems in the central nervous system (CNS) in MS, and its vulnerability to ROS effects may increase damage. Thus, treatment with antioxidants might theoretically prevent propagation of tissue damage and improve both survival and neurological outcome. Indeed, several experimental studies have been performed to see whether dietary intake of several antioxidants prevents or reduces the progression of EAE. Although a few antioxidants showed some efficacy in these studies, little information is available on the effect of treatments with such compounds in patients with MS. Well-designed clinical studies using antioxidant intake, as well as investigations based on larger cohorts studied over a longer periods of time, are needed in order to assess whether antioxidant intake together with other conventional treatments, might be beneficial in treating MS.

Key words multiple sclerosis · experimental autoimmune encephalomyelitis · oxidative stress · reactive oxygen species · antioxidants · blood brain barrier

Introduction

Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS), beginning most often in late adolescence and early adult life and characterized by perivenous infiltration of lymphocytes and macrophages into the parenchyma of the brain. MS can present in different forms, such as primary progressive (PPMS), relapsing progressive (RPMS), and secondary progressive phenotypes (SPMS) and relapsing remitting (RRMS), which is the most prevalent form (80 % of cases) [56].

Genetic factors such as interleukin-1 beta [63], interleukin-1 receptor [63], immunoglobulin Fc receptor genes [52], and apolipoprotein E (APO-E) gene [21] have been suggested as having a substantial effect on susceptibility to MS [62]. HLA-DR2, which was found to increase the risk of MS [36], and DQ polymorphisms are not associated with the course and severity of MS, despite their substantial contribution to disease susceptibility [78]. Environmental factors such pathogens and

chemicals and the high prevalence of MS in the northern hemisphere, [56] have also been suggested, and probably both genetic and environmental factors have a role in the pathogenesis of MS.

The etiology of MS has not yet been fully elucidated, but it is believed that immunological mechanisms are the most important in disease initiation and progression [68]. It is well known that pro-inflammatory cytokines, such as interferon γ and tumor necrosis factor β (TNF-β) released by activated TH-1 cells, may upregulate the expression of cell-surface molecules on neighboring lymphocytes and antigen-presenting cells. Binding of putative MS antigens, especially components of myelin such as myelin basic protein (MBP), myelin-associated basic glycoprotein (MOBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and others by the trimolecular complex, the T-cell receptor (TCR) and major histocompatibility complex (MHC) class II molecules on antigen-presenting cells, may trigger either an enhanced immune response against the bound antigens or anergy [56]. Indeed, autoantibodies against MBP and MOG have been found in MS patients [20]. In addition to the autoimmune response, oligodendrocyte death, axon damage and even neuronal loss have also been associated with the inflammatory attack on the CNS [17, 22, 65, 72, 73].

The therapy of choice for acute relapses in MS is based upon high dose, short-term pulse therapy with prednisolone or methyl prednisolone [50] and immunomodulatory treatments such as interferon beta-1a [9] and –1b [26], glatiramer acetate (copaxone®) [11], and mitoxantrone [15]. In addition, immunosuppression therapy with drugs such as azathioprine [82], cyclophosphamide [10], intravenous immune globulin (IVIG) [1] and additional therapies such as Plasmapheresis [79] have also been suggested. Other novel treatments requiring the evaluation of larger, placebo-controlled trials are estriol, [64] statins [54] and natalizumab, which is an α4 integrin antagonist [51].

Despite advances in MS pharmacotherapy, not all patients with MS respond well to treatment with these drugs, probably because of disease heterogeneity. Studies of patients with SPMS have yielded inconsistent results and, so far, there have been no positive phase III trials of immunomodulatory therapy in patients with PPMS.

Reactive free radicals and oxidative stress in multiple sclerosis

Biosynthesis of free radicals leading to oxidative stress

A free radical is any chemical species that contain one or more unpaired electrons. Unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical, because it acts as an electron acceptor and essentially "steals" electrons from other molecules. This loss of electrons is called oxidation, and free radicals are referred to as oxidizing agents because they tend to cause other molecules to donate their electrons. We are constantly exposed to free radicals created by electromagnetic radiation from the environment, both natural – (e. g., radon, cosmic radiation) and man-made (e. g., pollutants and cigarette smoke), and cellular metabolisms (e. g., respiratory burst, enzyme reactions). The most common cellular free radicals are hydroxyl radical ($OH^{\cdot}$), superoxide radical)($O_2^{\cdot-}$), and nitric monoxide ($NO^{\cdot}$). Other molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), are not free radicals but can lead to the generation of free radicals through various chemical reactions. Free radicals and related molecules are often classified together as reactive oxygen species (ROS) to signify their ability to lead to oxidative changes within the cell. Cells normally have a number of mechanisms to defend against damage induced by free radicals. Problems occur when production of ROS exceeds their elimination by the antioxidant defense system, or when the latter is damaged. This imbalance between cellular production of ROS and the inability of cells to defend against them is called oxidative stress (OS). OS can cause cellular damage and subsequent cell death because the ROS oxidize cardinal cellular components, such as lipids, proteins, and DNA (Fig. 1) [for review see 23].

Oxidative stress and excitotoxicity

Although multiple factors can precipitate OS in cells, the neurotransmitter, glutamate, is the major source of this process in the brain, primarily through activation of its

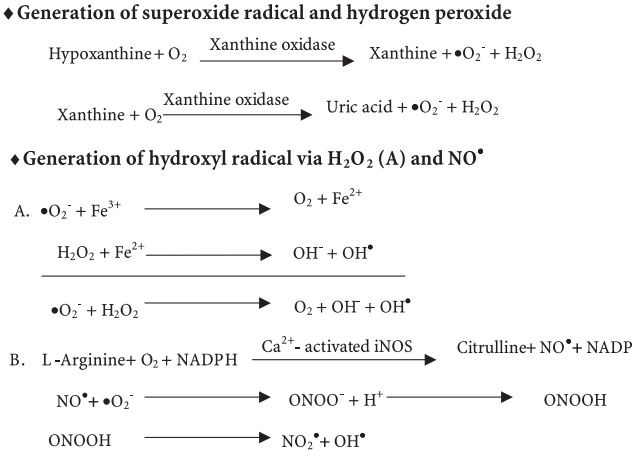

Fig. 1 Reactive oxygen species generation that may lead to cell death in MS (*NADP* nicotineamide adenine dinucleotide phosphate; *NADPH* reduced form of NADP; *iNOS* inducible nitric oxide synthetase)

ionotropic receptors. Glutamate and related excitatory amino acids account for most of the excitatory synaptic activity in the mammalian CNS and are released by an estimated 40% of all synapses. Ionotropic receptors can be distinguished by their pharmacological and electrophysiological properties: the N-methyl-D-aspartate (NMDA), which is the main glutamate receptor, α-amino-3-hydroxy-5-methyl-4-isoxasole-proprionic acid (AMPA) and the kainic acid (KA) receptors. The calcium-mediated effects of glutamate receptor activation, leading to neuronal degeneration, may involve a number of different pathways causing OS. Free radical-induced damage can occur by the stimulation of phospholipase A2 (PLA2) and the subsequent release of amino acids, which with oxygen radicals enhance the release of glutamate, thereby promoting a vicious circle [for review see 23].

Excitotoxic damage underlies part of the neurological deficits in EAE and MS. In-vitro studies suggest that glutamate, the major excitatory neurotransmitter in the mammalian brain, plays a role in MS pathophysiology. It was found that oligodendrocytes, the myelin-producing cell of the CNS, are highly vulnerable to glutamate excitotoxicity, mainly via the AMPA/kainate receptors [46, 48], which have higher permeability to Ca2+ [7]. Furthermore, demyelinating lesions caused by excitotoxins can be similar to those observed in MS, causing histologically similar damage [46–48].

Recent experimental studies showed that treatment with AMPA/kainate antagonists resulted in substantial amelioration of experimental autoimmune encephalomyelitis (EAE), the generally accepted animal model for the study of MS that has many clinical and pathological features of MS [56]. The AMPA/kainate antagonists also increased oligodendrocyte survival and reduced dephosphorylation of neurofilament H, an indicator of axonal damage [58, 67]. In one of these studies [58], mice acquired EAE after transfer of lymph node cells sensitized to myelin basic protein. The improvement in the clinical score observed in these animals following treatment with an AMPA/kainate receptor antagonist correlated with an increase in oligodendrocyte survival and reduced axonal damage. However, these beneficial effects were noted at an early stage of the disease, which does not usually involve demyelination. Another study [67], used the Lewis rat model of acute EAE, in which little demyelination occurs, and also the chronic EAE model. In both models, the secondary degeneration of spinal cord motor neurons, occurring as a consequence of the loss of white matter, was significantly reduced after treatment with glutamate receptor antagonists [67]. Unfortunately, neuronal versus oligodendroglial death and axonal loss were not examined simultaneously and no evaluation of demyelination was carried out. Therefore, the putative contribution of each type of damage to the clinical outcome cannot be assessed. Nevertheless, these findings open up new avenues for the treatment of autoimmune demyelination, such as riluzole, an antagonist of glutamate neurotransmission as a therapy administered for amyotrophic lateral sclerosis (ALS). Indeed, recently we have shown that riluzole, administered before and even after the appearance of clinical symptoms, dramatically reduced the clinical severity, inflammation, demyelination and axonal damage of MOG-induced EAE mice [24]. Consistent with this possibility, the neurological deficit resulting from EAE has generally been reduced by trial therapies to diminish the concentration of ROS [66].

There is also evidence in MS of an increase in cerebrospinal fluid (CSF) levels of glutamate in association with the severity and course of the disease [69, 3] and that glutamate production by macrophages may underlie axonal damage and oligodendrocytes death in MS lesions [80].

A potential cellular source that could contribute to enhanced glutamate levels in CSF is activated microglia, which are elevated during immunological attack and inflammation [55, 57]. These cells, in cultures exposed to high potassium, can release glutamate via reverse glutamate transport, a process tha is potentiated under pathological conditions [55]. Oxidative stress might also contribute to the increase in glutamate concentration in the extra-cellular space by reducing the efficiency of glutamate transporters [77].

Oxidative stress in the brain and neuronal tissue

ROS are particularly active in the brain and neuronal tissue as the excitatory amino acids and neurotransmitters, whose metabolism produces ROS, are unique to the brain as sources of OS. In addition, metals such as iron, catalytic for the free-radical reactions, are present in high concentrations in several regions of the brain, along with reduced transferrin levels in the CSF. Other sources are generated by constant use of oxygen in the mitochondria to supply the energy needs of these tissues. Free radicals are also produced by enzymatic pathways, such as xanthine oxidase, NADPH oxidase, lipoxygenases and cyclooxygenase, and by non-enzymatic mechanisms such as the auto-oxidation of catecholamines. ROS attack glia and neurons, which are post-mitotic cells and therefore are particularly sensitive to free radicals, leading to neuronal damage [for review see 23].

Oxidative stress, inflammation and demyelination in EAE and MS

It is well known that inflammation might raise ROS levels leading to OS. One of the most abundant sources of ROS, apart from the electron-transport chain of mito-

chondria, is the respiratory burst system of activated microglia. This system operates intermittently: when it is turned on, large quantities of ROS, especially superoxide ions)$O2^{-\cdot}$) are generated on the microglial external membrane, from which they are released into the surroundings. Other ROS includes hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), peroxynitrite (ONOO) and nitric oxide ($NO^{\cdot}$) the last named was shown to be produced by reactive astrocytes via cytokine-mediated induction of nitric oxide synthase [16].

Oxygen and nitrogen free radicals may be important in the pathogenesis of EAE and MS. Oxygen and nitrogen free radicals generated by macrophages have been implicated as mediators of demyelination and axonal injury in both EAE and MS [5, 38, 74]. In addition, free radicals can activate certain transcription factors, such as nuclear transcription factor-kappa B (NF-κB), which upregulate the expression of many genes involved in EAE and MS, such as tumor necrosis factor-α (TNF-α), nitric oxide synthase (iNOS), intracellular adhesion molecule 1 (ICAM-1) and vascular-cell adhesion molecule 1 (VCAM-1) [4, 81]. Also, redox reactions are involved in the activity of matrix metalloproteinases (MMP), which are important to T cell trafficking into the CNS [41, 49, 60].

Earlier studies found evidence of lipid peroxidation in the CSF [33], and in the plasma of patients with MS [53]. Numerous studies of patients with MS have shown increased free radical activity, and/or deficiencies in important antioxidant enzymes compared with healthy controls [42]. Two studies found reduced activity of the antioxidant enzymes, superoxide dismutase and glutathione peroxidase in red blood cells of MS patients [19, 83]. In another study, oxidative damage in the CNS was provoked by the release of iron from injured cells and low levels of both enzymatic (glutathione peroxidase), and non-enzymatic antioxidants, particularly ubiquinone and vitamin E, in the plasma and lymphocytes [70]. In addition, Karg et al. [37] found a 38 % increase in lipid peroxidation, significantly elevated levels of oxidized glutathione, and a reduction in the plasma vitamin E: lipid ratio during the active phase of MS. Examination of cerebrospinal fluid showed significantly higher concentration of isoprostanes [27] and increase of malondialdehyde (MDA) and glutathione reductase activity and decrease of glutathione peroxidase activity [8]. Moreover, direct examination of MS plaques has revealed increased free radical activity, along with decreased levels of important antioxidants such as glutathione, alpha-tocopherol and uric acid [39]. Furthermore, Vladimirova et al. [75] demonstrated that activated mononuclear cells of MS patients produce high amounts of ROS and nitric oxide and that oxidative damage to DNA, including mitochondrial DNA [43], develops in association with inflammation in chronic active plaques [76].

Different mechanisms have been proposed to explain how low levels of antioxidants or high levels of ROS might specifically mediate CNS damage in MS. Lower levels of antioxidants may promote increased activity of lipoxygenase, an enzyme which triggers the production of leukotrienes, thereby increasing the immuno-inflammatory processes in brain tissue [34]. Others have suggested that excess ROS can stimulate heightened T-cell activity via an arachidonic acid cascade, or produce direct/indirect damage to brain-blood barrier (BBB) or myelin [12].

Because of the pathogenic role of oxygen and nitrogen free radicals in MS pathology (Fig. 2), antioxidants might prevent free radical-mediated tissue destruction and inhibit some of the early pro-inflammatory events, such as T cell activation and trafficking into the CNS, that lead to inflammation and tissue destruction in EAE and MS.

Antioxidants in the treatment of EAE and MS

Antioxidants

Antioxidants are exogenous (natural or synthetic) or endogenous compounds acting in several ways including removal of O_2, scavenging ROS or their precursors, inhibiting their formation and binding metal ions needed

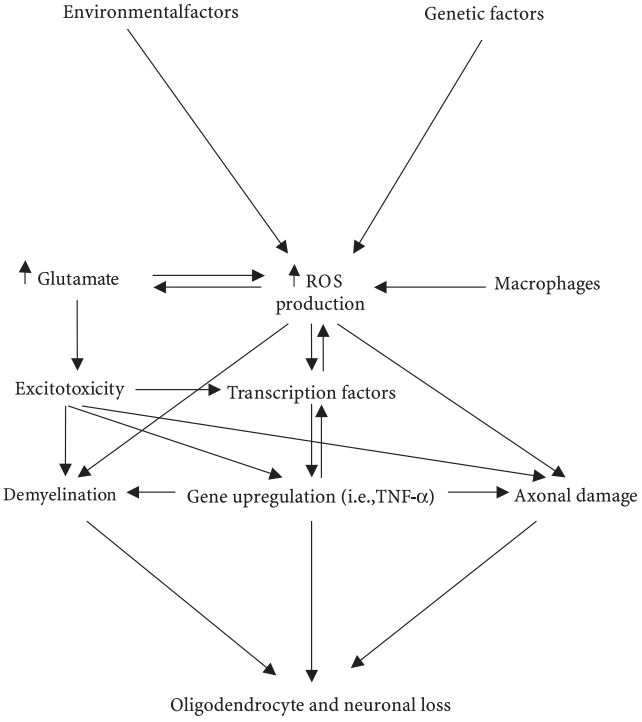

Fig. 2 The main sources of ROS and the cellular events that may lead to oligodendrocytes and neural loss in EAE and MS

for catalysis of ROS generation. The natural antioxidant system can be classified into two major groups: enzymatic (e. g., catalase, superoxide dismutase,) and non-enzymatic or the low molecular weight antioxidants (LMWA). The LMWA group of molecules can be further classified into directly-acting antioxidants (e. g., scavengers and chain breaking antioxidants) and indirectly-acting antioxidants (e. g., chelating agents). The former are extremely important in defense against OS, and most of them, including ascorbic and lipoic acids, polyphenols and carotenoids, are derived from dietary sources. The cell itself synthesizes a minority of these molecules, such as glutathione and NADPH.

The main problem in treatment of neurodegenerative diseases, including MS, is to introduce substances into the brain through the BBB, which is the major obstacle that separates the brain microenvironment from the blood within the cerebrovascular tree. This barrier reduces the efficacy of many agents, including antioxidants, owing to their limited passage through the BBB [for review see 23]. Therefore design of novel antioxidants that enables BBB transport will depend on new knowledge of BBB structure and transport processes.

Experimental studies

The encouraging findings with antioxidants in in-vitro studies led to their examination in experimental models of MS. Hall and colleagues [28] found that the antioxidant tirilazade mesylate, a member of the lazaroids family which were shown to scavenge lipid peroxyl radicals and inhibit iron-dependent lipid peroxidation, was effective in reducing the incidence and severity of actively-induced EAE. An additional study showed that oral administration of the oxidant-scavenger N-acetyl-L-cysteine (NAC), which can effectively raise intracellular glutathione levels, inhibited the induction of acute EAE. The protection was associated with enhancement of the specific lymphocyte proliferative response to the immunizing antigens (spinal cord homogenate) at early stages, post encephalitogenic injection [40]. Another investigation showed that intra-peritoneal administration of catalase before the onset of neurological deficit delayed the onset of EAE and reduced its severity and duration [61]. Furthermore, butylated hydroxyanisole, another LMWA, was effective in reducing the incidence, severity and mortality of passively induced EAE [29]. A synthetic salen-manganese complex, Euk-8, may be regarded as a prototype molecule of a new class of synthetic catalytic scavengers with combined SOD and catalase activity, was also shown to ameliorate EAE in mice [44]. The authors showed that repeated injection of Euk-8 starting at the time of EAE induction delayed the onset and markedly reduced the severity of the disease. In addition, all Euk-8-treated mice completely recovered after 40 days. Moreover, the authors showed that post treatment with Euk-8, 4 days after EAE induction, also resulted in a significant amelioration of EAE [44]. Recently, it was shown that alpha lipoic acid (-lipoate (thioctic acid, 1.2-dithiolane-3-pentanoic acid; 1.2-dithiolane-3 valeric acid; and 6.8-dithiooctanoic acid) a well known antioxidant, supplied in the diet and capable of crossing the BBB, inhibited EAE in mice. It was shown that alpha lipoic acid had dose-dependent reduction in the 10-day cumulative disease score, and suppressed inflammation, demyelination and axonal damage in the spinal cord. Moreover, there was a marked reduction in CD3 + T cells and CD11b + monocyte/macrophage cells within the SC, and inhibition of matrix metalloproteinase-9 (MMP-9) activity in a dose-dependent fashion. The authors' conclusion was that alpha lipoic acid suppressed EAE probably by inhibiting T cell trafficking into the spinal cord, perhaps by the inhibition of MMP-9 activity [45]. Current data from our laboratory show that administration of N-acetylcysteine amide (AD4), our novel BBB-penetrating low-molecular antioxidant [2], prior or even after the emergence of symptoms, significantly reduced the clinical severity of MOG-induced EAE C3H.SW mice [25]. In addition, it also reduced inflammation, axonal damage and inhibited MMP-9 activity [25] probably in a mechanism that involves, at least in part, macrophage-induced ROS inhibition.

Several investigators have shown that inhibition of the inducible/inflammatory form of iNOS, as well as the use of anti-sense knockdown of iNOS, suppress EAE in mice and rats [6, 14, 18, 84]. Moreover, Hooper et al. [31, 32] demonstrated that the inhibition of iNOS, or scavenging of $NO^{\cdot}$ or peroxynitrite by uric acid, inhibited neurological deficits in mice with EAE [31, 32], while withdrawal of iNOS inhibitor resulted in the appearance of neurological signs within 24 hours [31, 32]. In contrast, recent data showed that inhibition of NO production is lethal [13]. Thus, it is still not clear whether NO inhibition is beneficial in treating EAE.

Clinical trials

Following the encouraging findings in the EAE models, many authors have suggested that dietary antioxidant intake i. e., vitamin E or selenium may help to inhibit disease progression [12, 34, 70]. Indeed, Jensen et al. [35] showed that supplementation with antioxidants (6.6 mg. of sodium selenite, 2 gm of vitamin C and 500 I. U. of vitamin E per day) increased and normalized the glutathione peroxidase activity and the cellular content of linoleic acid in erythrocytes and hematogenous cells within 3 weeks, with no effect on MS severity.

However, despite media reports of individuals who appear to benefit from following restrictive regimens, there is minimal scientific evidence that antioxidant in-

take is effective in slowing the rate or severity of MS. Only two studies of mild to severely disabled subjects have been performed to date, suggesting that the degree of MS disability did not correlate significantly with intake of specific nutrients [30, 71]. The authors' explanation for this phenomenon was that people with severe disability or poor care support would be unlikely to participate in such studies.

Conclusions

Several findings suggest that OS might be involved in the pathogenesis of EAE and MS, and that antioxidant administration may be useful in prevention and treatment of EAE. However, very few clinical studies have been performed to determine whether antioxidant intake might truly protect or slow down the progression of MS. To obtain efficacy in delaying MS progression, the candidate antioxidant must be given as early as possible, before oligodendrocyte and irreversible neuronal loss. It also should be tailored to the precise OS physiology, e. g. the type of ROS involved, the place of generation, and the severity of the damage. In addition, the chosen antioxidant should be able to penetrate the BBB after systemic administration in order to attain a critical therapeutic level within the CNS. Thus, antioxidant cocktails or those combined with the conventional therapies e. g., immunosuppressors or immunomodulator drugs, might have more successful synergistic effects. Well-designed clinical studies using antioxidant intake, as well as observational investigations based on larger cohorts studied, over a longer period of time, are needed in order to examine whether antioxidant intake might be beneficial for MS therapy.

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