The Role of Iron in the Pathogenesis of Experimental Allergic Encephalomyelitis and Multiple Sclerosis

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ABSTRACT: Multiple sclerosis (MS) and its animal model, experimental allergic encephalomyelitis (EAE), are autoimmune disorders resulting in demyelination in the central nervous system (CNS). Pathologically, the blood-brain barrier becomes damaged, macrophages and T cells enter into the CNS, oligodendrocytes and myelin are destroyed, astrocytes and microglia undergo gliosis, and axons become transected. Data from several biochemical and pharmacological studies indicate that free radicals participate in the pathogenesis of EAE, and iron has been implicated as the catalyst leading to their formation. The primary focus of this article is the examination of the role of iron in the pathogenesis of MS and EAE. Particular attention will be paid to the role and distribution of iron and proteins involved with iron metabolism (e.g., transferrin, ferritin, heme oxygenase-1, etc.) in normal and disease states of myelin. Furthermore, therapeutic interventions aimed at iron, iron-binding proteins, and substrates or products of iron-catalyzed reactions leading to free radical production will be discussed.

KEYWORDS: iron; myelin; oligodendrocyte; multiple sclerosis; experimental allergic encephalomyelitis; oxidative damage; heme oxygenase; ferritin; transferrin

INTRODUCTION

In humans, lipids represent ~33% of the dry weight of gray matter, ~55% of the dry weight of white matter, and ~70% of the dry weight of myelin.[1] The oligodendrocyte is responsible for producing massive quantities of lipids that become incorporated into the multilamellar structure of myelin, and each oligodendrocyte can produce up to 50 or more myelin segments. This "lipid factory" requires sufficient enzymatic machinery for the biosynthetic steps required for this high level of lipid production, and many of these enzymes utilize iron as part of their catalytic center. It has been suggested that the high concentration of iron observed within the oligo-

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dendrocyte is due to an elevated expression of enzymes involved with myelin production, although other explanations have been put forth, such as the oligodendrocyte being a center for iron distribution to the rest of the central nervous system (CNS). In addition to partaking in normal physiological processes within oligodendrocytes, the high levels of iron have been suggested to promote pathogenesis during disease states such as multiple sclerosis (MS) and its animal model, experimental allergic encephalomyelitis (EAE), due to the ability of iron to catalyze reactions that lead to oxidative tissue damage. The function of high levels of iron within oligodendrocytes in healthy states and the role of iron in demyelinating diseases of the CNS are the focus of this paper.

IRON, TRANSFERRIN, AND FERRITIN IN OLIGODENDROCYTES AND MYELIN

A large number of studies have examined the distribution of iron in the CNS by histochemical staining procedures. The Perls' histochemical stain has been used in many investigations, especially in combination with 3,3′-diaminobenzidine enhancement of the ferric ferrocyanide reaction product.[2] Other modifications include the utilization of permeabilization steps to increase the penetration of histochemical reagents into densely myelinated areas[3,4] and changes in fixatives and/or incubation times.[5] A consensus among many studies is that iron is enriched within oligodendrocytes and myelin.[3,4,6–12] Electron microscopic studies revealed iron deposits in the cytoplasm of oligodendrocytes[11] and within the inner and outer loops of myelin,[6] and it is possible that compact myelin also contains appreciable amounts of iron.[4] In addition to iron histochemical staining, substantial concentrations of iron also have been detected by atomic absorption in myelin fractions of brain homogenates.[13]

The role that high levels of iron perform within oligodendrocytes is not fully established. The high concentration of iron in oligodendrocytes has been suggested to be associated with biosynthetic enzymes that are involved with the high metabolic demands of myelinogenesis.[3,14] However, phylogenetic studies revealed that oligodendrocytes in the fish and frog did not have high iron levels as detected by histochemistry,[15] suggesting that high iron levels are not essential for the formation and/or maintenance of myelin. On the other hand, iron deficiency during early postnatal life causes a reduction in myelination,[16,17] indicating that the oligodendrocyte is sensitive to low iron levels. An alternate suggestion was that, in species with iron-enriched oligodendrocytes, the iron serves as a storage depot to be tapped for delivery of iron to other cells in the CNS. In support of this idea is the observation that oligodendrocytes synthesize transferrin,[12,18–20] and this transferrin may deliver iron to other cells in the CNS.[21] In one study, cultured oligodendrocytes were shown to synthesize and secrete transferrin,[22] but this was not confirmed in a follow-up study.[23] Thus, it is uncertain whether iron delivery by oligodendrocyte transferrin contributes significantly to the transport of iron in the CNS. Oligodendrocyte transferrin also has been suggested to serve in an autocrine capacity to help oligodendrocytes accumulate iron.[24] However, transferrin receptors are absent or present in low abundance in white matter,[25,26] and mechanisms to sequester iron other than involving transferrin can be used by glial cells.[27] Transferrin has been shown to serve as a growth factor[28] and it was found to be important for the maturation and function of oligodendro-

cytes,[29–31] suggesting that the receptor may be present in oligodendrocyte progenitors. However, the role of transferrin as a growth factor would not account for the large accumulation of iron that is observed within oligodendrocytes.

Unlike transferrin receptors, ferritin receptors are present in high concentrations in white matter,[32,33] and cultured oligodendrocytes bind and internalize ferritin.[34] Furthermore, due to the large binding capacity of ferritin for iron, there is the potential for a greater delivery of iron by ferritin than transferrin.[32] In addition to binding ferritin, oligodendrocytes express ferritin.[12,35–37] There are two subunits of ferritin, heavy (H) and light (L), and both subunits are expressed by oligodendrocytes.[38–41] Neurons, microglia, and astrocytes also express ferritin similar to oligodendrocytes,[40,42] yet these cells are not routinely stained by iron histochemistry, suggesting that the large accumulation of iron observed in oligodendrocytes is not necessarily due solely to the presence of ferritin. Moreover, immunohistochemical staining of ferritin or transferrin fails to reveal staining of myelin. This is in contrast to findings with iron histochemistry where myelin staining has been clearly documented.[3,4,6–12] Thus, it is likely that there is no one protein that accounts for the large majority of iron binding in oligodendrocytes. Transferrin, ferritin, and iron-containing enzymes involved with myelinogenesis probably all contribute to sites of iron localization within oligodendrocytes. The proteins that bind iron within myelin are less clear. However, iron enrichment within both oligodendrocytes and myelin raises the possibility that an imbalance in the management of iron during disease could lead to the production of iron-catalyzed free radicals that result in oxidative damage.

IRON, FERRITIN, AND TRANSFERRIN IN EAE AND MS

Histochemical staining of iron in CNS tissue from SJL mice with EAE revealed iron deposits that were not present in the CNS of normal animals. For example, during clinically active disease, there was histochemical staining of iron within macrophages and extravasated RBCs, and granular staining was present in extracellular sites and possibly within some astrocytes.[43] During the recovery phase of disease, staining persisted in macrophages and granular deposits.[43] In tissue from MS patients, an initial report by Craelius *et al*.[44] revealed abnormal iron deposits in 5 out of 5 MS patients, but these findings were not fully confirmed in two subsequent studies.[45,46] These three studies on MS tissue did not include any steps to enhance the permeability of the tissue to the histochemical reagents, and they were carried out on paraffin sections where the processing steps could facilitate the leaching of iron from the tissue. When Vibratome sections were utilized together with permeabilization steps, iron deposits were observed in macrophages in tissue from 5 out of 5 MS patients, and labeled reactive microglia and ameboid macrophages were observed in 3 out of 5 tissues.[47] In tissue from 1 MS patient, labeling of axons was present.[47] Craelius *et al*. also noted axonal staining in their report.[44] It is possible that the axonal staining revealed axons that were recently transected since axonal transection is a predominant pathological feature of MS.[48–51] In addition to labeled axons, punctate iron deposits were observed within some neurons of patients with MS similar to that observed for neurons in CNS tissue from patients affected with Alzheimer's disease.[47] These deposits within neurons likely represent cells undergoing degeneration since neuronal loss is also a pathological feature of MS.[52] In addition to neurons, punctate deposits

were observed within some oligodendrocytes in MS tissue.[47] Mitochondria are possible sites of these punctate deposits within oligodendrocytes and neurons since two proinflammatory cytokines found in MS, TNF-α and IL-1β, have been shown to lead to the accumulation of iron within mitochondria in astrocyte cultures.[53]

During stress, such as hypoxia, oligodendrocytes increase their synthesis of ferritin,[54,55] and ferritin levels are increased in the CNS of EAE animals compared to control animals.[56] Ferritin levels, but not transferrin or iron levels,[57,58] were found to be significantly elevated within the CSF of MS patients with chronic progressive active disease, but not relapsing remitting disease, compared to levels in the CSF of control patients.[58] In other conditions, the upregulation of ferritin expression is thought to be associated with the protection of cells against oxidative damage[59–62] and/or the inhibition of cell-mediated immunity.[63–67] Cell-mediated immunity, that is, T cells and macrophages, is the major contributor to pathology in EAE and MS. Thus, the elevated levels of ferritin in EAE and MS may be a protective mechanism to limit the toxic effects of iron during ongoing pathogenesis.

In MS tissue, there is an absence of ferritin binding sites in and adjacent to lesion sites, which is likely due to the loss of oligodendrocytes in this disease, while in the normal brain there is a relatively high concentration of ferritin receptors in white matter compared to gray matter.[33] Unlike ferritin, transferrin can bind to periplaque regions and to occasional plaques in MS tissue,[33] indicating that the receptors accounting for transferrin binding were present in cells other than oligodendrocytes.

Natural resistance–associated macrophage protein-1 (Nramp1) modulates iron metabolism in macrophages and is thought to play an important role in macrophage activation.[68] Since the macrophage is critical for the pathogenic development of EAE and MS, Nramp1 has been suggested to be involved with CNS demyelinating diseases. Although far from proven, an allele of this gene has been suggested to be associated with MS susceptibility in South African Caucasians[69] and alleles in this gene might influence the susceptibility and/or severity of other autoimmune diseases such as rheumatoid arthritis.[70,71] Thus, the management of iron in the CNS may be a precipitating factor for the onset and/or progression of MS.

FREE RADICAL DAMAGE TO OLIGODENDROCYTES/MYELIN

The abnormal iron deposits observed in EAE and MS tissues indicate that the normal homeostasis of iron is disrupted, and iron is likely released from the proteins that it normally binds. Released iron will quickly bind to neighboring molecules, and iron that is loosely bound, or in a free state, can catalyze reactions that lead to the production of reactive oxygen intermediates (ROI). ROI can promote cellular damage at many levels, for example, proteins, DNA, lipids, mitochondrial function, etc. Data from a variety of studies indicate that oxidative tissue damage occurs in EAE and MS. For example, lipid peroxidation products were observed in EAE and MS tissue,[72,73] the production rates of ROI from inflammatory cells were increased from EAE mice compared to control mice,[74,75] and pharmacological interventions aimed at disrupting oxidative damage have therapeutic value in EAE and possibly MS (discussed below).

During EAE or MS, cells in the CNS respond to inflammation by inducing the expression of stress response proteins.[76,77] One stress response protein related to

iron metabolism is heme oxygenase-1 (HO-1). HO-1 expression can be induced by many factors including heme, metals, glutathione depletion, nitric oxide, cytokines, etc., and several of these stressors are present in EAE and MS. For example, abnormal iron deposits are present in EAE and MS tissues,[43,47] glutathione depletion occurs in EAE and MS,[56,78,79] nitric oxide and its products are increased in EAE and MS,[80,81] and proinflammatory cytokines are enhanced in MS.[82,83]

HO-1 acts in association with NADPH cytochrome P450 reductase, which provides reducing equivalents, to convert heme into biliverdin, carbon monoxide, and iron. The various products of HO-1 activity have pro- or antioxidative properties.[84] For example, biliverdin and bilirubin (which is rapidly generated from biliverdin by biliverdin reductase) are both antioxidants. The released iron is a pro-oxidant if it is not properly sequestered. Cells protect themselves from the toxic effects of iron by responding to an increased iron load by downregulating the transferrin receptor[85] and by upregulating ferritin expression.[86,87] Lower levels of transferrin receptor will restrict the entry of additional iron into the cell, while the increased expression of ferritin will bind and store the released iron. Iron-responsive proteins (IRPs) bind iron-responsive elements on ferritin mRNA, preventing its translation.[86,87] When IRPs sense an increase in cytosolic iron levels, they allow ferritin mRNA to undergo translation, which results in the sequestering of iron by ferritin. Disruption of this regulation can lead to an enhanced accumulation of iron and ubiquitin-containing inclusions within oligodendrocytes.[88] Furthermore, mice deficient in H-ferritin have increased evidence of oxidative stress in their CNS.[89] Thus, a coordinated response is required to limit the potential toxic effects of iron that is liberated by HO-1.

HO-1 expression has been observed to be increased in EAE[90,91] and MS[53] tissues, and immunohistochemical studies revealed that HO-1 was expressed predominantly by macrophages and some astrocytes in EAE tissue[90,91] and in astrocytes in MS tissue.[53] The administration of the HO-1 inhibitor, tin-protoporphyrin IX, to EAE mice resulted in the enhanced induction of HO-1 levels above the levels already increased by the EAE disease.[56] HO-1 induction was not observed in control animals given tin-protoporphyrin IX, suggesting that the breakdown of the blood-brain barrier that occurs in EAE[92,93] accounts for the greater access of drugs to the CNS in EAE animals compared to control animals,[94] and this would allow tin-protoporphyrin IX to induce the expression of HO-1 in CNS cells, similar to what has been observed in the liver.[95] Tin-protoporphyrin IX appeared to enhance the expression of HO-1 in astrocytes and microglia since these cells were only occasionally observed in EAE animals given vehicle, but they were observed more frequently in EAE animals given tin-protoporphyrin IX (FIGS. 1A–C). In addition to greater expression in astrocytes and microglia, induction was also enhanced in radial glia (FIG. 1D) and possible other cells as well. Besides inducing HO-1 expression, tin-protoporphyrin IX is known to inhibit HO-1 activity, especially at higher doses (discussed below).

During EAE, the oligodendrocyte is exposed to stress due to the inflammatory response directed at its antigens. However, HO-1 staining was not evident in this cell even after the administration of tin-protoporphyrin IX to EAE animals. Even though HO-1 staining was not clearly observed within oligodendrocytes, all cells need to metabolize heme. Thus, oligodendrocytes should have the capacity to express one or more types of HO, which could facilitate the deposition of iron in ferritin, whose expression is elevated in this cell type[38–41] and whose levels increase in the CNS during EAE.[56] The inability to detect HO-1 staining in oligodendrocytes during

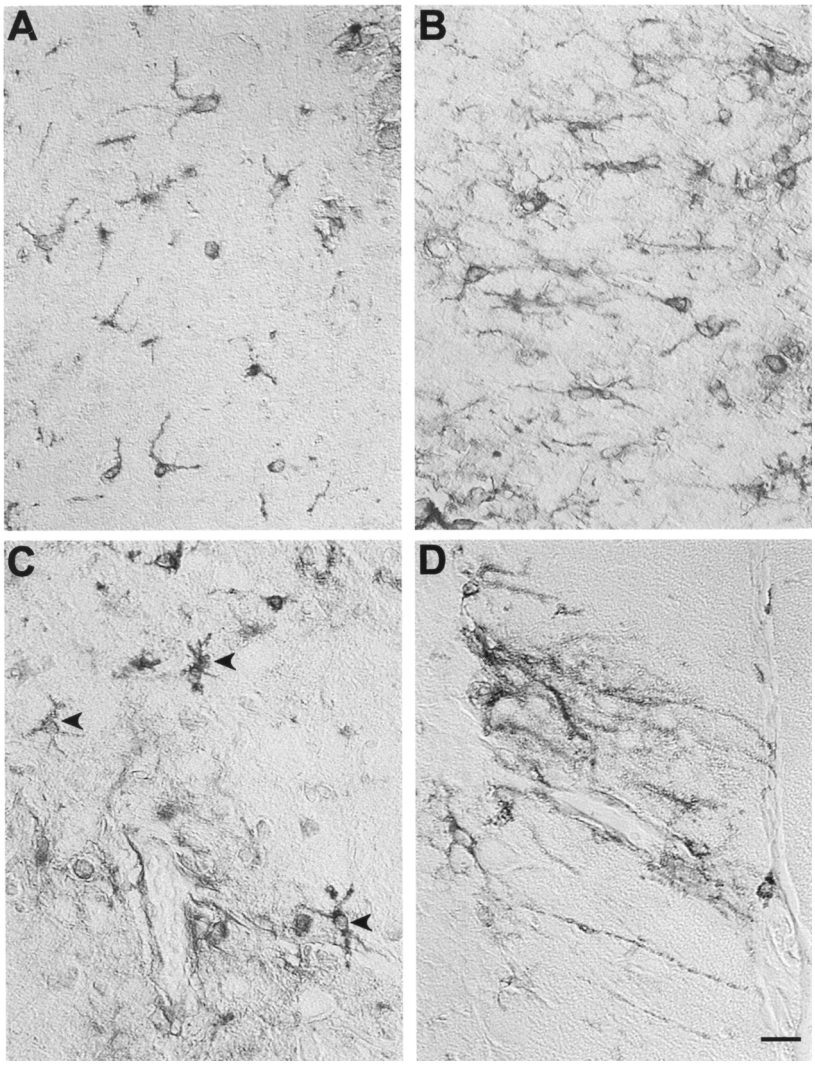

FIGURE 1. HO-1 immunohistochemical staining on formalin-fixed, paraffin sections utilizing 1:6000 rabbit anti-mouse HO-1 (StressGen Biotechnologies, Victoria, British Columbia, Canada), horseradish peroxidase–labeled goat anti-rabbit IgG, and 3,3′-diaminobenzidine. The frequency of labeling in **(A)** reactive microglia, **(B)** ameboid microglia, **(C)** astrocytes (*arrowheads*), and **(D)** radial glia appeared greater in EAE SJL mice given 50 μmol/kg (C, D) or 200 μmol/kg (A, B) tin-protoporphyrin IX than EAE mice given vehicle, suggesting that tin-protoporphyrin IX could induce HO-1 expression in the CNS of EAE animals. Labeled infiltrating, round macrophages were abundant in EAE animals given vehicle or tin-protoporphyrin IX (not shown). Bar: 20 μm.

disease could be due to several factors. For example, the levels of expression could be well below that for other cells, such as macrophages, which would make it difficult to optimize staining conditions that clearly reveal staining in both cell types. Alternatively, a different form of HO than HO-1, for example, HO-2, could be expressed by oligodendrocytes or the expression of HO-1 could last only minutes or hours and thus missed in a disease lasting several days for EAE and many years for MS patients.

As HO-1 expression increases during disease, the overall expression of NADPH cytochrome P450 reductase expression decreases in EAE tissue.[90] The reduction of NADPH cytochrome P450 reductase would appear to be inconsistent with the increase of HO-1 in the CNS of EAE animals; however, this enzyme is also used by other enzymes such as the cytochrome P450s. The activity of a cytochrome P450 was found to be reduced in the CNS of EAE animals,[96] which would parallel the reduction of NADPH cytochrome P450 reductase,[90] and other cytochrome P450s also may be reduced. Furthermore, the reduction in NADPH cytochrome P450 reductase levels is consistent with its pattern of expression in other models of stress.[97,98] Thus, while NADPH cytochrome P450 reductase is likely associating with HO-1 during EAE, on balance its levels throughout the brain are reduced during disease.

THERAPEUTIC APPROACHES TARGETING PATHOGENIC MECHANISM INVOLVING IRON

Various therapeutic interventions targeting iron, iron management, or iron-catalyzed free radicals have been explored for the treatment of EAE and MS. The most direct approach has been the utilization of iron chelation therapy. In 1984, Bowern *et al*.[99] administered the iron chelator, Desferal (also known as desferrioxamine and deferoxamine), to Lewis rats given guinea pig spinal cord homogenates as the encephalitogen. Both the duration and severity of disease were reduced in the treated groups. In a subsequent study, Desferal failed to reduce disease severity in Lewis rats given myelin basic protein (MBP) as the encephalitogen, but the drug was administered only from days 1–7 postencephalitogen injection, while disease onset was day 11.[100] In an effort to clarify the discrepancy between these two studies, a third study was performed on SJL mice given MBP as the encephalitogen.[94] Desferal was given during the clinical stage of disease rather than the preclinical period as was the case in the second negative study.[100] Treatment with Desferal resulted in disease suppression in this third study, and immunohistochemical staining of Desferal revealed its presence in the CNS of EAE animals.[94] Administration of Desferal during the active stages of experimental uveitis was also found to suppress lipid peroxidation in the retina.[101] Thus, taken together, these data support the notion that Desferal acts to suppress the active stage of disease by limiting iron-catalyzed free radical tissue damage.

Due to the ability to inhibit free radical tissue injury, Desferal was tested for therapeutic value in three studies on MS patients. In the first study, 12 MS patients were given 2 g/day for 5 days/week for 3 months.[102] At the end of the study, 7 of the 12 patients showed improvement, 4 patients were unchanged, and 1 was worse. The second study gave Desferal at 2 g/day for 7 days followed by 1 g/day for an addi-

tional 7 days. At 3 months following treatment, 9/18 patients showed improvement, 7/18 were unchanged, and 2/18 showed worsening; however, as time progressed, the patients displayed a trend to have a worsening of disease.[103] In the third study, Desferal was given at 2 g/day for 7 days followed by 1 g/day for an additional 7 days and this was repeated every 3 months for 2 years. Out of 9 patients, 1 showed improvement, 3 were unchanged, and 5 worsened by 0.5 points on the Kurtzke expanded disability status scale.[104] Taken together, the results are inconclusive about whether Desferal has therapeutic value for the treatment of MS, and a larger, double-blind trial needs to be performed to resolve this question. Although the patients appeared to tolerate Desferal reasonably well, a serious drawback is that this drug is usually administered by a subcutaneous pump over several hours, which is a difficult and cumbersome method for drug administration. A more promising approach may be the administration of an iron chelator that can be given by an oral route when a suitable one becomes available.

Although there is debate as to whether HO-1 serves a protective or pathogenic role during disease,[84,105] interventions aimed at HO-1 have been pursued for the treatment of EAE. Hemin (40 μmol/kg), an inducer of HO-1 expression, was found to ameliorate EAE in Lewis rats, while tin-mesoporphyrin (40 μmol/kg), an inhibitor of HO-1, was found to worsen disease.[106] The authors suggest that HO-1 suppresses disease by the production of biliverdin, which is converted to bilirubin, and/or carbon monoxide.[106] Bilirubin serves as an antioxidant,[85,107,108] while carbon monoxide is thought to be an anti-inflammatory agent.[109] In a follow-up study, bilirubin was administered (50, 100, 200 mg/kg) to Lewis rats during the active stage of EAE and it was found to suppress disease in a dose-dependent manner.[110] However, caution should be exercised with respect to advancing this form of therapy for a chronic condition like MS since high levels of bilirubin can adversely affect the nervous system.

A second study examined the role of HO-1 in EAE.[56] In this study, the SJL mouse model was used together with the HO-1 inhibitor, tin-protoporphyrin IX (50 and 200 μmol/kg). The high dose of inhibitor was found to suppress clinical and pathological evidence of disease, and oxidative stress was reduced.[56] In the SJL model, there is extravasation of RBCs into the CNS during disease,[43,111] and the release of iron from heme by HO-1 has been suggested to account for the pathogenic effects of HO-1 in this model, which may be similar to pathogenic mechanisms of HO-1 in stroke,[112] traumatic brain injury,[113] and cerebral ischemia.[114] Thus, the different results between studies on Lewis rats and SJL mice may be due to variations in pathological features between these models or the different doses of inhibitors. Since pathological studies indicate variations of pathological mechanisms in MS,[115] and extravasation of RBCs has been suggested in some MS patients,[46,116] it is unclear whether HO-1 serves to advance or attenuate pathology during the course of this disease.

The propathogenic mechanism of HO-1 in SJL mice with EAE was suggested to be related to a large release of iron from heme and a failure of ferritin to adequately sequester the iron in a timely and/or complete manner.[56] Thus, to test this possibility, apoferritin was administered to SJL mice with EAE. Apoferritin was found to suppress disease activity in EAE mice, while injections of iron, which increased serum ferritin levels, failed to ameliorate the disease course.[117] It was suggested that the ferritin synthesized in response to iron injections quickly acquired the injected iron and lost some or most of its therapeutic potential since ferritin loaded with iron can release iron, especially when exposed to superoxide anion radical or nitric oxide.[118,119] The

therapeutic action of apoferritin was suggested to be due to the sequestering of the exogenous iron that occurs in this disease,[43] and this mechanism would be similar to that suggested for Desferal described above.

Other therapies aimed at reducing the substrates or products of iron-catalyzed reactions leading to ROI have been tested in EAE. The administration of catalase, but not superoxide dismutase, to Lewis rats with EAE resulted in the suppression of disease activity.[75] High doses of uric acid, a scavenger of peroxynitrate, which is produced from superoxide anion radical and nitric oxide, resulted in suppression of disease in PLSJL mice.[120] Scavengers of oxygen radicals such as α-lipoic acid,[121] butylated hydroxyanisole,[122] EUK-8,[123] melatonin,[124] *N*-acetyl-L-cysteine,[125] thymoquinone,[126] etc., resulted in disease suppression. Thus, there is a growing body of evidence indicating that interventions aimed at iron or at the substrates or products of iron-catalyzed reactions that produce ROI ameliorate EAE disease. These studies suggest that this pathogenic mechanism may hold potential as a target for therapeutic intervention for MS.

SUMMARY

The massive quantities of lipids produced by oligodendrocytes may be responsible, in part, for the high accumulation of iron in this cell type. Some proteins that are involved with iron management, for example, ferritin and transferrin, are also expressed in abundance within this cell. Disruption of iron metabolism within oligodendrocytes, or in other cells within the CNS, could help to precipitate or advance MS, and interventions targeting iron-catalyzed reactions warrant further exploration for the treatment of MS.

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