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New Insights into S-nitrosylation in Multiple Sclerosis

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Abstract

S-nitrosylation is a biologically relevant post-translational protein modification with signaling consequence. In eukaryotes, a large number of proteins have been identified as S-nitrosylation targets. Derangement in protein S-nitrosylation has been implicated in the pathogenesis of a number of different disease entities including Multiple Sclerosis (MS). A growing body of evidence has shown that Nitric oxide (NO) plays a critical role in MS. NO and other reactive nitrogen species (RNS) are involved in neuroinflammation and neurodegeneration in MS. Signaling by RNS is carried out mainly by S-nitrosylation of critical cysteine residues in targeted proteins. In recent years, newer roles in MS have been attributed to RNS. These roles relate to S-nitrosylation of cysteines in proteins which has emerged as a potential new paradigm in signal transduction and regulation of protein function. In the present review we discuss the evidence for the diverse roles of S-nitrosylation in MS, including nitrosative stress-induced gene expression in MS, and S-nitrosylation of transcription factors in MS. In addition, S-nitrosylation can be therapeutically used in MS. Recent studies providing evidence for SNO-based therapy strategy in the treatment of MS will also be discussed. Undoubtedly, new exciting results will contribute to the expanding area of MS research.

Keywords: Multiple sclerosis; S-nitrosylation; Nitric oxide; iNOS; Transcription factor; Biomarkers; Therapy; Posttranslational modification; Autoimmune disease

Introduction

Nitric Oxide (NO) has long been recognized as a modulator of gene expression both in prokaryotic and eukaryotic cells and is an import molecule involved in many physiological and pathological processes [1,2]. NO is synthesized by Nitric Oxide Synthase (NOS) which oxidizes a guanidine nitrogen of L-arginine releasing nitric oxide in the form of a free radical and citrulline. Three isoforms of the NOS have been identified, including neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3). S-nitrosylation is one of the key mechanisms by which NO regulates the function of various target proteins is through the coupling a nitroso moiety from NO-derived metabolites to a reactive cysteine leading to the formation of a S-nitrosothiol (SNO) [3]. SNOs are stable, bioactive forms of NO and are known to regulate the immune response [4]. Classic NO signaling delineates a pathway by which NOS-derived NO diffuses to and then binds to the heme moiety of guanylate cyclase inducing a conformational change that results in enzyme activation and increased formation of cyclic GMP (cGMP) [5]. Nitrosative stress has been implicated in the pathophysiology of MS and its animal model experimental autoimmune encephalomyelitis (EAE) [6-8]. It was reported that protein SNOs accumulate in the brain of MS patients and SNO levels are also increased in EAE [6,9].

MS is a chronic inflammatory demyelinating disease of the central nervous system (CNS), which is the most frequent disabling neurological disease in young adults. MS afflicts over 2 million people worldwide. According to the temporal course of disease, MS can be subdivided into three clinical groups: relapsing remitting MS (RR-MS), secondary progressive MS (SP-MS) and primary progressive MS (PP-MS). Most evidence supports that the activation of autoreactive T-cells is a central event in the development of autoimmune response in MS and the pathogenesis of MS in most patients is likely to result from autoreactive, activated $CD4^+$ T cells moving from the periphery across the blood brain barrier (BBB) into the CNS [10]. There are numerous symptoms associated with the neurologic damage in MS patients, including fatigue, spasticity, depression, bowel and bladder dysfunction, pain, and impaired mobility. Several therapies (eg. modafinil, dalfampridine, baclofen, diazepam, gabapentin, and opioids) are used for symptomatic treatment of disability and symptoms, but these do not improve disease outcome [11]. This chronic immune-mediated disease potentially requires more definitive symptomatic and disease-modifying therapies.

Nitrosative stress induces the generation of protein and non-protein nitrosothiols, resulting in alterations in tissue function [12,13]. Accumulating evidences points to an important role for NO in the pathogenesis of MS and to its contribution to the various facets of the disorder: inflammation, oligodendrocyte injury, changes in synaptic transmission, axonal degeneration, and neuronal death [14]. Boullerne et al. found that S-nitrosothiols was detected in MS patients and EAE animals [15,16]. Calabrese et al. also reported that the concentration of both nitric oxide metabolites and unidentified low molecular weight nitrosothiols were increased in serum and cerebrospinal fluid (CSF) from patients with active MS [17]. Recent studies have reported that SNOs accumulate in brain white matter of MS patients, indicating that the occurrence of protein S-nitrosylation correlates with the inflammatory demyelinating disorders in MS patients [8]. This review paper provides insights into the role of protein S-nitrosylation in the pathophysiology of MS and summarizes the SNO-based therapy strategy in the treatment of MS.

Nitrosative Stress-induced Protein S-nitrosylation in MS

NO has been linked to numerous physiological and pathophysiological events. It is very important to identify the protein targets of S-nitrosylation which include metabolic, structural and signalling proteins. Previous studies indicated that protein S-nitrosylation acts as a physiological signalling mechanism in MS [18].

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Cytoskeletal proteins

Jaffrey et al. reported that the proteins which were S-nitrosylated comprise neurofilament heavy chain (NFH), α/β-tubulin, and β/α-actin when the rat cerebellum homogenates were incubated with NO donors *in vitro* experiments [18]. *In vivo*, S-nitrosylation of both α-tubulin and β-tubulin was increased only during acute EAE [19]. The S-nitrosylation of the major microfilament protein β-actin was also detected only in animals with acute EAE [20], but β-actin were not modified by S-nitrosylation in either control or EAE tissues as well as dynein, ankyrin, and tropomyosin. It is likely that the abnormal S-nitrosylation of several structural proteins such as NFPs, tubulin, and β-actin in EAE may contribute to the pathophysiology of MS.

Proteolipid protein (PLP)

Exposure to NO donors causes myelin decompaction, accompanied by S-nitrosylation of a cysteine-rich proteolipid protein (PLP) [7,14]. Indeed, incubation of rat spinal cord slices with GSNO resulted in the S-nitrosation of a number of proteins [6,21]. In myelin, one of the major S-nitrosated substrates was identified as PLP, an abundant cysteine-rich protein that is responsible for the intraperiod line (IPL) stabilization [6]. It is proposed that NO-mediated nitrosation of sulfhydryl groups is likely to interfere with the normal function of PLP and other important CNS myelin proteins leading to the structural demise of this membrane. These findings are relevant to multiple sclerosis and other inflammatory demyelinating disorders where both excessive NO production and myelin instability are known to occur [7]. S-nitrosylation of PLP has been linked to decompaction of CNS myelin at the level of the intraperiod line, where this protein plays an adhesive role.

Metabolic enzymes

In vitro experiment, incubation of rat spinal cord slices with GSNO leads to S-nitrosylation of four metabolic enzymes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), creatine kinase (CK), hexokinase 1 (HK), and glycogen phosphorylase (GP) [18]. Among the four metabolic enzymes that are S-nitrosylated in vitro, only GAPDH was S-nitrosylated to a higher level in EAE *in vivo* experiment. The S-nitrosylation of GADH Cys-149 at the active site significantly attenuates the activity of this glycolytic enzyme [22,23]. It was also found that GSNO inhibited GAPDH activity in both purified enzyme preparations and endothelial cells [24]. In addition, recent studies indicated that the S-nitrosylation of GAPDH induces its binding to the E3 ubiquitin ligase Siah1 to cause nuclear translocation and to promote apoptosis [25]. GSNO induced S-nitrosylation of HK causes enzyme inactivation, but the effect apparently is caused by S-nitration of several tyrosine residues instead of by S-nitrosylation of cysteine thiols [26]. GSNO also inhibits the activity of Triosephosphate isomerase (TPI), Phosphofructokinase (PFK), Neuron-specific enolase (NSE), GP and Creatine kinase (CK) through S-nitrosylation [27-29]. *In vivo* experiment, TPI, PFK, and GP were S-nitrosylated to the same extent in control and EAE tissues. Bizzozero and Zheng reported that NSE is heavily modified in acute EAE and is minimally S-nitrosylated in control spinal cords [6]. CK was barely modified in control and EAE spinal cord [29,30]. These finding suggest that S-nitrosylation of important metabolic enzymes such as GAPDH and NSE could lead to neuronal death later in the disease process in MS.

Ion-channels-related proteins

The NO donors induced the S-nitrosylation of 3 ion channel-related proteins including N-methyl-d-aspartate (NMDA)-glutamate receptors, hyperpolarization activated cation channel (HCN3), and Na1/K1 ATPase α-2 subunit [18]. *In vivo*, HCN3 was not S-nitrosylated either in control or in EAE tissues. Na/K ATPase α-2 subunit was modified equally in control and EAE spinal cords. In contrast, the proportion of S-nitroso-NR2A increased in both acute and chronic EAE [6]. Choi et al. reported that S-nitrosylation of a single cysteine residue in NR2A modulates its channel activity [5]. Site-directed mutagenesis identified a critical cysteine residue (Cys 399) on the NR2A subunit whose S-nitrosylation under physiological conditions underlies this modulation. Bizzozero et al. found that the proportion of S-nitrosylated NMDA receptors increased in EAE [6]. They also discovered that neuronal specific enolase is the major S-nitrosylated protein in acute EAE. Given that S-nitrosylation affects protein function, it is likely that the observed changes are significant to the pathophysiology of inflammatory demyelination in MS [6]. The NMDA receptor (NMDAR)-associated ion channel was modulated not only by exogenous NO but also by endogenous NO. In cell systems expressing NMDARs with mutant NR2A subunits in which this single cysteine was replaced by an alanine, the effect of endogenous NO was lost [5]. Thus endogenous S-nitrosylation can regulate ion channel activity.

Signal transduction proteins

Jaffrey et al. found that the retinoblastoma (Rb), heat-shock protein 72 (Hsp72), isoforms 2 of the collapsin-response-mediator protein (CRMP2), and calbindin were S-nitrosylated when rat cerebellum homogenates were incubated with the NO donors GSNO [18]. *In vivo*, Rb protein was not detected in either the total homogenate of mouse Spinal cords (T1-L5). HSP-72, CRMP-2, and calbindin were detected in the total homogenates of mouse Spinal cords (T1-L5) [6]. These findings indicate that, although some proteins are susceptible to S-nitrosylation *in vitro* with various NO donors, they may not be modified *in vivo* to any appreciable extent even under severe nitrosative stress conditions.

S-nitrosylation of Transcription Factors in MS

NF-κB

NF-κB is a transcription factor activated by cell surface receptor signaling to meet stress and inflammatory responses, regulating key cellular processes such as inflammation, innate and adaptive immunity, and cell growth and survival [31]. Accumulating evidences indicate that NF-κB plays an important role in controlling expression of genes relevant to the pathogenesis of autoimmunity. Genetic factors related to NF-κB may also be determinants of MS susceptibility [32]. Within chronic active MS lesions and adjacent white matter, both NF-κB and c-jun/JNK reactivity was markedly up-regulated on glial cells and inflammatory elements [1]. NF-κB p50-deficient mice were significantly resistant to EAE induced by myelin oligodendrocyte glycoprotein. The resistance to EAE in NF-κB p50-deficient mice was associated with a deficiency of myelin oligodendrocyte glycoprotein-specific T cells to differentiate into either Th1- or Th2-type effector cells *in vivo*, suggesting that NF-κB plays crucial roles in the activation and differentiation of autoreactive T cells *in vivo* and that blocking NF-κB function can be an effective means to prevent autoimmune encephalomyelitis [2]. NO acts as second messenger molecular which through S-nitrosylation has been shown to control important cellular processes by regulation of activity of NF-κB [33]. NF-κB activity is exquisitely sensitive to cellular NO levels with multiple steps in the signaling pathway targeted by S-nitrosylation. In addition, both p50 and p65 have been shown to be targeted by S-nitrosylation in cytokine-stimulated respiratory epithelial cells [34]. In addition to direct modification of NF-κB proteins, NO can also alters NF-κB activity through S-nitrosylation of proteins in other

signal transduction pathways that cross-talk with NF-κB [34]. NO inhibits TLR-4 activation of NF-κB via S-nitrosylation of MyD88 [35]. Based on the role of NF-κB in MS, S-nitrosylation of NF-κB could be considered as a new therapeutic target in MS.

HIF

Hypoxia-inducible factor (HIF) is a transcription factor that regulates cellular hypoxic responses, and it has therapeutic potential in MS. An increased expression of HIF-1α in MS normal-appearing white matter (NAWM) in oligodendrocytes was detected by in situ hybiridization analysis and quantitative RT-PCR [36]. HIF-1α, a key regulator of hypoxia-induced gene regulation, and its downstream genes were significantly unregulated in MS NAWM in the microarray study [37]. The upregulation of HIF-1α in oligodendrocytes supports the view of oligodendrocyte and/or neuronal dysfunction in the NAWM as a possible primary cause. These studies suggest an endogenous inflammatory reaction throughout the whole white matter of MS brain, in which oligodendrocytes actively participate. Recent studies also demonstrate that HIF stabilization and transcriptional activity is achieved through S-nitrosylation of HIF pathway components [38]. HIF-1 plays a critical role in the mammalian program by which cell respond to hypoxia in both physiological and pathological situations. HIF-1 transcriptional activity, protein stabilization, protein-protein interaction, and cellular localization are mainly modulated by post-translation modifications such as hydroxylation, acetylation, phosphorylation, S-nitrosylation, and SUMOylation [39]. Under normal oxygen tension, HIF-1 activity is usually suppressed due to the rapid, oxygen-dependent degradation of HIF-1α. Normoxic HIF-1 activity can be upregulated through NO-mediated S-nitrosylation and stabilization of HIF-1α [40].

IRF

Interferon regulatory factor (IRF) family is a group of transcription factors that are induced following treatment with type I interferon (IFN) [41]. Following the initial identification of two structurally related members, IRF-1 and IRF-2, seven additional members have now been reported [42]. IRF-1 is an interferon-induced transcription factor with pro-inflammatory and pro-injurious functions. New evidences emerged over past decade indicated that IRF-1 gene is associated with progressive MS and the elevated expression of IRF-1 was detected in active and chronic-active MS lesions [43-45]. IRF-1 was detected in the areas of CNS inflammation and co-localized with the perivascular mononuclear cells as well as with microglia and oligodendrocytes [46]. Oligodendrocyte injury and inflammatory demyelination are key pathological abnormalities of MS and its animal model EAE. Emerging evidences indicate that oligodendrocytes can regulate the events leading to inflammatory demyelination [47]. The role of IRF-1 in EAE was initially investigated using IRF-1 KO mice. In these studies, the KO mice were found to be resistant to EAE upon immunization with MOG 35-55 compared to wild-type mice [48,49], indicating suppression of IRF-1 signaling resulted in a dramatic protection against EAE without any appreciable adverse effects. IRF-1 appears to be directly involved in the pathogenesis of MS, oligodendrocyte injury, and inflammatory demyelination. It suggests that IRF-1 acts as a master transcription factor orchestrating oligodendrocyte injury and inflammatory demyelination in MS and EAE. New evidences also show that IRF-1 regulates the autophagic response in LPS-stimulated macrophages through NO. *In vivo*, tissue macrophages obtained from LPS-stimulated IRF-1 knockout (KO) mice demonstrated increased autophagy compared to those isolated from IRF-1 wild-type mice [50]. In vitro, LPS-stimulated peritoneal macrophages obtained from IRF-1 KO mice experienced increased autophagy. IRF-1 mediates the inhibition of autophagy by modulating the activation of the mammalian target of rapamycin (mTOR). The inhibitory effects of IRF-1 mTOR activity were mediated by NO [48]. Herein, we propose a novel role for IRF-1 and NO-induced S-nitrosylation in the regulation of MS. In addition, recent findings suggest that IRF-4 is essential for the development and function of T helper (Th) cell, regulatory T (Treg) cell, B cell, as well as dendritic cell (DC) and these cells are crucial in the pathogenesis [49]. Functional studies have provided evidence that Th17 cells are important for the modulation of autoimmune responses in MS, and Th17 cells are controlled by IRF4 [51], suggesting that IRF-4 also contributes to the pathogenesis of MS. Currently, although there is no direct evidence to show that S-nitrosylation involve in the function and regulatory mechanism of IRF-4 in MS, future investigation may provide new evidence about Nitrosative Stress-induced IRF-4 regulation in MS. In addition, Tregs cells also play a vital role in MS. Brahmachari and Pahan reported that NO inhibited the expression of Foxp3 in MBP-primed T cells via soluble guanylyl cyclase-mediated production of cGMP, indicating a novel role of NO in suppressing Foxp3(+) Tregs via the soluble guanylyl cyclase (sGC) pathway in MS [52,53]. sGC is the major cellular receptor for the intercellular messenger NO and mediates a wide range of physiological effects through elevation of intracellular cGMP levels [54].

SNO-based Therapy Strategy in the Treatment of MS

Multiple sclerosis is the most frequent chronic inflammatory, demyelinating and neurodegenerative disease in young adults, but has no definitive pharmacological treatment. Most therapeutic agents used in MS including immunosuppressive and immunomodulatory drugs and cell cycle interruption drugs are only used for the treatment of RR-MS. These therapeutic agents can lessen the relapse rate in RR-MS and time to progression, but cannot cure MS. Therefore, there is a need for new efficient treatments for all types of MS. A more definitive therapy for MS should reduce relapse rate, prolong remission, limit the onset of new MS lesions, and postpone the development of long-term disability. There is a growing interest in developing a treatment strategy focused on protein posttranslational modification in MS, including S-nitrosylation. Herein, we are drawing attention to S-nitrosylation as a potential therapeutic strategy in MS (Figure 1).

The S-nitrosylated protein biomarkers for detection of MS

The symptoms of MS include independent processes of inflammation, demyelination, neurodegeneration, gliosis and repair. The progress made in the search for new biomarkers in MS is helpful for the early diagnosis, prognosis, evaluation of the development of the disability caused by the disease and the response to therapy [55]. Biomarkers are very helpful to make decision in clinical diagnostics and important for guiding therapeutic treatment. MS is a class of disorders that need early diagnosis and steady monitoring. Now it was confirmed that S-nitrosylation affects the immunogenicity of self-protein antigens, and triggering an autoimmune response. In this context, S-nitrosylated peptides provided a more valuable tool with respect to isolated or recombinant proteins to selectively detect autoantibodies as disease biomarkers. It is now well established that some posttranslational modifications can generate new self-antigens or even mask antigens normally recognized by the immune system in physiological conditions. The most extensively studied putative self-antigens are components of normal myelin of the central nervous system, or of their post-translational modified forms [55]. Peptides can

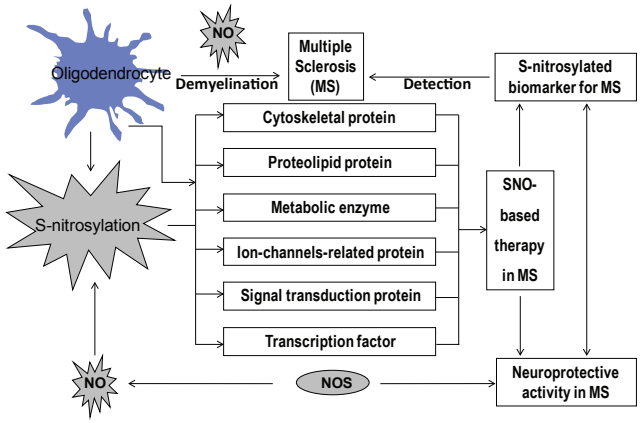

Figure 1: The role of S-nitrosylation in Multiple Sclerosis (MS). Protein SNOs accumulate in the brain of MS patients and SNO levels are also increased in EAE, indicating that the occurrence of protein S-nitrosylation correlates with the inflammatory demyelinating disorders in MS patients. Oligodendrocyte injury and inflammatory demyelination are key pathological abnormalities of MS and its animal model EAE. The S-nitrosylation target proteins include cytoskeleton proteins, proteolipid protein, metabolic proteins, ion channel proteins, signaling proteins, and transcription factors. Then S-nitrosylation of proteins could be a potential therapeutic strategy in MS. The S-nitrosylated protein biomarkers are helpful for the early diagnosis, prognosis, evaluation of the development of the disability caused by the disease and the response to therapy. Activation of iNOS and NO generation was identified as a marker and therapeutic target in neuroinflammatory conditions in MS.

be used as synthetic antigenic sources to mimic the native antigens especially when S-nitrosylation is supposed to trigger the autoimmune response. In this perspective, the S-nitrosylated protein biomarkers can contribute to the development of diagnostic/prognostic immunoassays particularly useful to guide therapeutic treatments in MS.

Neuroprotective activity of S-nitrosylation in MS through the regulation of NOS

S-nitrosylation can be therapeutically used in MS. The main focus of study on the role of NO in MS has been the iNOS isoform because it is the high-output form of NOS and can produce several orders of magnitude more NO than eNOS or nNOS [33]. iNOS has been found to be a major contributor to initiation/exacerbation of CNS inflammatory conditions through the production of excessive NO which generates RNSs. iNOS expression is mainly controlled at the level of transcription and can be induced by an appropriate combination of cytokines in almost every cell type [33]. Recent studies have reported that MS and EAE are resulting from an increase iNOS [6,9]. Activation of iNOS and NO generation were identified as a marker and therapeutic target in neuroinflammatory conditions in MS. The positive modulators of iNOS gene expression include the redox sensitive transcription factors NF-κB, HIF-1α, and the small GTPase Ras [34,56-58]. The S-nitrosylation of the p50 or p65 subunit of NF-κB inhibits its DNA binding activity, resulting in the repression of iNOS expression [59,60]. On the contrary, S-nitrosylation-caused inhibition of IκB prevents its phosphorylation and dissociation from NF-κB, thus prevents the nucleus translocation of NF-κB [59]. Inhibition of NF-κB activation initiates a proinflammatory response and also suppresses iNOS expression. Investigators in search of iNOS modulating pharmacological agents have realized the need of a delicate balance so as to allow the production of physiologically relevant amounts of NO but at the same time block the generation of RNSs through repressing excessive NO levels [33]. Recent studies provided the prevalent strategies at the transcriptional levels, which the post-translational modification of iNOS might define, its activity for pharmacological modulation. iNOS seems to establish a link between neuroendocrine and immune system through beta-endorphin explaining stress-related relapses in MS [61]. Understanding the modulation of iNOS and NO production may provide better therapeutic strategies for MS.

Concluding Remarks

S-nitrosylation is a biochemical modification that plays an important regulatory role in signal transduction in MS. Accumulating evidences showed that functional changes resulting from the S-nitrosylation, and the growing number of proteins were shown to be S-nitrosylated in MS. Currently, drug development for MS faces numerous challenges with many drugs failing at various stages of development [62]. In addition, a number of agents are in development, but thus far no beneficial agent has been established in primary-progressive MS [33]. A more definitive therapy for MS should reduce relapse rate, prolong remission, limit the onset of new MS lesions, and postpone the development of long-term disability. Detailed studies addressing the role of S-nitrosylation in MS by endogenous NO and RNS are not abundant, but those that are available pave the way for future developments. These findings raise the intriguing possibility that S-nitrosation is directly involved in the modulation of protein function [63]. It could be expected that more and more research has been focused on the control of physiological levels of NO and for the design of new drugs that inhibit pathological induction of iNOS to prevent overproduction of NO for the treatment of MS.

Herein, we review recent progresses in the field of S-nitrosylation and MS research that may have direct implications for our understanding of the role of S-nitrosylation in MS-associated pathology and for designing SNO-based therapies for MS. Recent discoveries highlight the need to investigate the protein targets of S-nitrosylation in MS and the discovery of the S-nitrosylated transcriptional factors and relevant proteins opens an unexplored signaling realm with great potential for therapy. It is the aim of this review to provide new insights into the role of S-nitrosylation and the therapeutic modulation of iNOS and NO production. This review hopes to serve as a summary of the prevalent strategies in the regulation of protein function in MS.

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