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REVIEW

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The role of nitric oxide in multiple sclerosis

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Abstract During the past decade nitric oxide has emerged as an important mediator of physiological and pathophysiological processes. Elevated nitric oxide biosynthesis has been associated with nonspecific immune-mediated cellular cytotoxicity and the pathogenesis of chronic, inflammatory autoimmune diseases including rheumatoid arthritis, insulin-dependent diabetes, inflammatory bowel disease, and mutiple sclerosis. Recent evidence suggests, however, that nitric oxide is also immunoregulatory and suppresses the function of activated proinflammatory macrophages and T lymphocytes involved in these diseases. This article reviews the role of nitric oxide in the biology of central nervous system glial cells (astrocytes and microglia) as it pertains to the pathogenesis of multiple sclerosis in humans and experimental allergic encephalitis, the animal model of this disease. Although nitric oxide has been clearly implicated as a potential mediator of microglia-dependent primary demyelination, a hallmark of multiple sclerosis, studies with nitric oxide synthase inhibitors in the encephalitis model have been equivocal. These data are critically reviewed in the context of what is know from clinical research on the nitric oxide pathway in multiple sclerosis. Specific recommendations for future preclinical animal model research and clinical research on the nitric oxide pathway in patients are suggested. These studies are necessary to further define the role of nitric oxide in the pathology of multiple sclerosis and to fully explore the potential for nitric oxide synthase inhibitors as novel therapeutics for this disease.

Key words Multiple sclerosis · Nitric oxide · Microglia · Astrocytes · Demyelination

Abbreviations *BDV* Borna disease virus · *CSF* Cerebrospinal fluid · *EAE* Experimental allergic encephalomyelitis · *EPR* Electron paramagnetic resonance · *IFN* Interferon · *IL* Interleukin · *LPS* Lipopolysaccharide · *MBP* Myelin basic protein · *MS* Multiple sclerosis · *NF* Nuclear factor · *NOS* Nitric oxide synthase · *RT-PCR* Reverse-transcriptase polymerase chain reaction · *TGF* Transforming growth factor · *TNF* Tumor necrosis factor

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Introduction

Multiple sclerosis (MS) is an autoimmune, inflammatory, demyelinating disease in which myelin and the myelin-producing cells, oligodendrocytes, are destroyed. Myelin, the multilayered membrane surrounding the axon, is an active participant in nervous system function, and nerve conduction slows when myelin is disrupted. In MS plaque tissue oligodendrocytes appear to be dying in a necrotic fashion: they are hypertrophied, with swollen nuclei and disrupted plasma and mitochondrial membranes. The prevalence of actively phagocytosing cells in the plaques suggests destruction of myelin by activated macrophages and suggests that free radicals of oxygen and nitrogen are at work in this pathology.

It is clear that nitric oxide (NO•) is able to modulate the induction of the immune response, permeability of the blood-brain barrier, trafficking of cells to the central nervous system, and local responses in the inflammatory milieu. In this regard it might be predicted that NO• could be both beneficial and harmful in an autoimmune disease such as MS. This brief review explores both the hypothetical and the scientifically demonstrated relationship between NO• and plaque formation in the CNS of MS brains, with a discussion of the intervening steps involving cytokines, adhesions molecules, and vascular changes at the blood-brain barrier. The contributrion ends with a summary of the evidence in MS, in the animal model experimental allergic encephalomyelitis (EAE), and in an in vitro model of oligodendrocyte damage and death, for the presence and possible function of NO• in preventing and/or perpetuating the disease process.

Cytokines in MS and EAE

It is clear that proinflammatory cytokines such as interferon (IFN) γ, interleukin (IL) 1, and tumor necrosis factor (TNF) α or ligands for the Fc receptor and complement receptor (C3biR) can activate macrophages and glia to produce free radicals of oxygen and nitrogen in the rodent [1]. While proinflammatory cytokines do not directly activate adult human macrophages and glia to produce NO•, they most likely amplify NO• production incurred by additional membrane perturbations or the cross-linking of cell surface molecules other than cytokine receptors [2–4]. In this regard proinflammatory cytokines could play a role in NO• production in the peripheral immune system as well as in the CNS.

It is very clear now from a variety of studies examining proinflammatory cytokines at the protein and/or mRNA levels in MS patients' blood plasma, cerebrospinal fluid (CSF), brain tissue, and cultured blood leukocytes that among other proinflammatory cytokines, IFN-γ, IL-1, and TNF-α are elevated (reviewed in [5, 6–12]). TNF-α and IFN-γ rises seem to predict a relapse in MS, and the number of circulating IFN-γ positive blood cells correlates with moderate to severe disability [10–13]. IL-1 is constitutively produced by MS patients' lymphocytes in vitro in the absence of stimulation, suggesting in vivo activation [8]. Indeed, in clinical trials treatment of MS patients with IFN-γ exacerbated the disease [14, 15] while treatment with IFN-β improved patients' clinical scores [16]. It is believed that IFN-β may benefit MS patients by inhibiting IFN-γ inducible major histocompatibility antigen class II (MHCII) genes, among others [17]. Interestingly, the mRNAs for transforming growth factor TGF) β and IL-4, cytokines which can be both pro- and anti-inflammatory, are also elevated in MS patients' blood cells as determined by in situ hybridization [10].

As has been postulated for MS, EAE is a $CD4^+$ T cell dependent, Th1-mediated disease in which MHCII is elevated on macrophages and microglia. Early (even preclinically) in EAE, IL-2, IL-2R, and IFN-γ are all elevated, while IL-4 and TNF-α appear at the height of clinical disease in the brains and spinal cords of these animals [18]. The presence of IL-10 in brain tissue is correlated with recovery from disease [19]. Interference with TNF-α or IL-1 function in vivo in EAE animals by use of specific antibodies to either the ligand or receptor, soluble receptors, or receptor antagonists ameliorates the disease [20]. Physical or functional elimination of macrophages from EAE-susceptible animals prevents disease induction [21]. Pretreatment of EAE animals before disease induction with anti-inflammatory cytokines such as IL-13 or IL-10 inhibits the disease [22–24] while IL-4 enhances the disease in one model [22]. IL-13 inhibits both clinical and histological signs in EAE and may do so through its capacity to downregulate IL-1, TNF-α, and NO• production by macrophages and microglia [23]. In a separate approach to inhibiting EAE, Khoury et al. [25] have demonstrated that myelin basic protein (MBP) induced oral tolerance is associated with the upregulation of TGF-β, IL-4, and prostaglandin E. Both MHCII and TNF-α production, elevated before the oral tolerance, are downregulated in these myelin-fed animals.

These studies provide evidence for an association between activated macrophages and microglia and their cytokine products with the inflammatory loci in both MS and EAE CNS tissue. In the case of EAE it is clear from the studies cited that the clinical and histological findings are dependent on the macrophage as an effector cell. Therefore the next question is: how do the cytokines which are incriminated in lesion formation regulate NO•?

Cytokine regulation of NO •

Table 1 summarizes numerous studies from the literature which examine NO• formation and nitric oxide synthase (NOS) enzyme expression in primary rodent and human glial cells and cell lines in vitro. Much of the regulation of NO• production in macrophages and glia has been studied in the rodent. In humans such cells are clearly turned on to produce the type II, or inducible, NOS-2 and NO• when certain cell surface receptors are cross-linked; these include CD4, a non-CD4 binding site for HIV-1, CD23, and CD69 [2–4, 26]. The role of cytokines in augmenting NO• induction in human glia and macrophages in vitro may be very different from that in rodent cells in vitro and even different from effects in vivo in human disease [26, 27].

Table 1 NOS isoform expression in rodent and human glial cell cultures

Cell type, species	Isoform	Stimuli	References
Primary rat astrocytes	NOS-1	Glutamate, norepinephrine, NMDA, dexamethasone	124–125
Primary rat astrocytes	NOS-2	LPS, S-100β	126–132
Primary rat microglia	NOS-2	LPS, IFN-γ, TNF-α, IL-2, β-amyloid	28, 130, 133–135
Rat C6 glioma cells	NOS-2	LPS, IFN-γ, TNF-α, IL-1β	126, 136–139
Primary mouse astrocytes	NOS-2	IFN-γ, IL-1β	140–141
Primary mouse microglia	NOS-2	LPS, IFN-γ	142–144
Mouse microglial cell lines	NOS-2	LPS, IFN-γ, TNF-α, zymosan	145
Primary human astrocytes	NOS-2	LPS, IFN-γ, TNF-α, IL-1β	115–117, 119, 146
Primary human microglia	NOS-2	LPS, IFN-γ, TNF-α, IL-1β	115–117, 147
Human astrocytoma cells	NOS-2	LPS, IFN-γ, TNF-α, IL-1β	148–151

NO• inducers: IFN-γ, TNF-α, and IL-1b

IFN-γ is a strong inducer of NOS-2 and NO• in rat glia and macrophages, and this production is synergistically increased in the presence of TNF-α or lipopolysaccharide (LPS; reviewed in [1, 27–29]). About half of the LPS-induced, but not IFN-γ induced, NO• is mediated through TNF-α [28]. IFN-γ and IL-1b, but not LPS, are inducers of NOS-2 and NO• in human glial cells derived from fetal brain tissue [26, 30] (Fig. 1), but not from neonatal or adult brain-derived glial cells ([1, 26] and this laboratory's unpublished observations). Stevenson et al. [31] have shown that IL-12, probably through the induction of TNF-α and IL-1β, induces NO•. IL-6 has no effect on NO• induction [32–34] nor does IFN-β alone [35–37]. Nevertheless, mouse macrophages primed by suboptimal doses of LPS are triggered by IFN-β to produce NO• [37, 38].

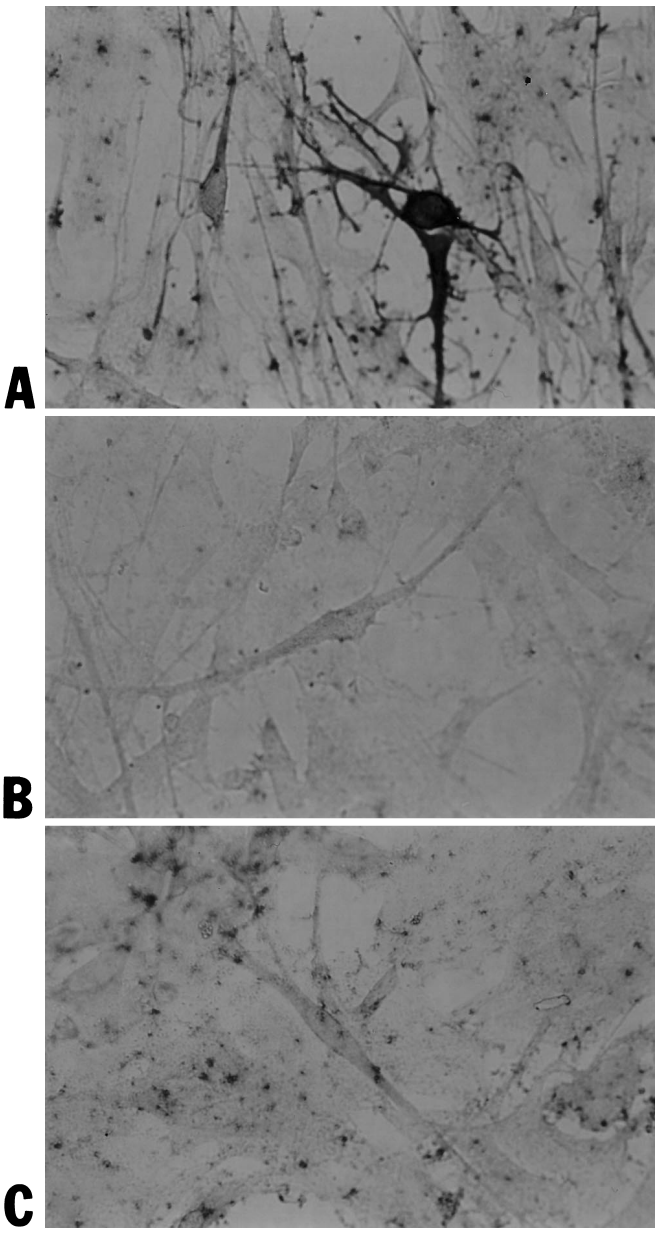

Fig. 1A–C Human fetal glial cells stained for NOS-2. **A** Polyclonal rabbit anti-human hepatocyte NOS-2 antibody (1:100; gift of Dr. David Geller, University of Pittsburgh) was used to stain second-trimester human fetal microglia and astrocytes which were stimulated for 72 h with 500 U/ml human recombinant IFN-γ and 300 U/ml human recombinant IL-1b. **B** The stimulated human glia as in **A**, stained with normal rabbit serum at 1:100. **C** Human glia which have not been induced with cytokines stained with anti-human hepatocyte NOS-2 as in **A**

NO• modulators: IL-13, TGF-β, IL-4, and IL-10

Since it is clear that certain cytokines block EAE, and that NO• is involved in EAE (see below), it is of interest to know whether blockade of NO• by these same cytokines accounts for their inhibition of EAE. Doyle et al. [39] report that IL-13 suppresses NO• production but not respiratory burst and oxygen radicals in murine macrophages; this cytokine also suppresses TNF-α, although to a lesser extent. Such a finding suggests a mechanism for the inhibition of development of EAE by IL-13 [23]. It also demonstrates the independence of oxygen and nitrogen free radical production in these cells. This is an important observation given that $ONOO^-$ may cause greater damage in cells and tissues than NO•, but that both O_2^{-}• and NO• need not be inhibited at the same time for an effect by an anti-inflammatory cytokine. In cultures of rodent macrophages and microglia it is almost universally accepted that TGF-β inhibits NOS-2 and NO• through several mechanisms [28, 40–45]. These include inhibition of transcription and translation of NOS-2, decreased stability of NOS-2 mRNA, increased degradation of NOS-2 protein, and stimulation of arginase, thereby depleting the enzyme substrate L-arginine [42–44, 46, 47].

The effects of IL-4 and IL-10 on NO• production are not as straightforward as the aforementioned cytokines. The capacity of IL-4 and IL-10 to inhibit NOS-2 and NO• appears to depend on pretreatment of the cells being stimulated with IFN-γ; activated cells are not as easily or not at all inhibited by IL-4 and IL-10 [48–59]. The mechanism for this inhibition has been variously suggested as through the reduction of TNF-α [53, 60, 61], inhibition of the activation of protein kinase Cε [58], or induction of arginase [62]. These cytokines may synergize with each other or with TGF-β [45, 50]. Still other studies have found modest or no inhibition of NO• [1, 63] or an increase in NO• by IL-10 [47].

Depending on the state of activation of human monocytes/macrophages, IL-4 may up- or downregulate NO•. IL-4 can directly induce NOS-2 and NO• production in resting monocytes from normal healthy human donors [64–67]. This activation can be amplified by pretreatment with IFN-γ [65]. In spontaneously high NO• producer monocytes, especially those from allergy patients, IL-4 abrogates NO• [64]. IL-4 has also been shown to induce NO• in murine splenocytes [68]. The production of elevated NO• in allergy patients and the ability of IL-4 to induce NO• indirectly in peripheral blood mononuclear cells is

probably related to the fact that cross-linking of the molecule CD23, the low-affinity IgE Fc receptor (FcεRII), by IgE or anti-FcεR antibody leads to NO• [4, 69–71]. IL-4 induces IgE production as well as an increase in soluble CD23 [69, 70]. Interestingly, high levels of IL-4 and NO• may ultimately feedback negatively, possibly through the elevation of cAMP, on both IgE production and NO• [69–71] which may explain the IL-4 inhibition of "spontaneously" produced NO• from allergy patients [64].

In cells which have not been stimulated by cytokines or LPS, a brief rise in intracellular cAMP leads to a small but significant direct induction of NOS-2, as well as leading to the amplification of NOS-2 and NO• by subsequent cross-linking of CD23 [72]. However, in cells stimulated by TNF-α, IL-1β, or LPS, prolonged elevation of cAMP, via adenyl cyclase activators (e.g., prostaglandin E_2), phosphodiesterase inhibitors or β-adrenergic agonists, inhibits NO• production [1, 73, 74]. Phosphodiesterase inhibitors such as pentoxifylline, isobutyl-methylxanthine, and iloprost variously inhibit TNF-α production, cytotoxicity, and O_2^{-}• production in addition to NO• production [74–77], possibly through the inhibition of nuclear factor (NF) κB which is involved in NO• and TNF-α induction [78]. Data supporting a role for both TNF-α and NO• in EAE are therefore complemented by studies demonstrating that the phosphodiesterase inhibitors pentoxifylline and rolipram inhibit EAE in rodents and primates, most likely through the inhibition of cytokines, inflammation, demyelination, and NO• [79–81].

NO • regulation of cytokines

In some autoimmune diseases, including MS, a pernicious proinflammatory cycle may account for the clinical and histopathological chronicity. In this regard it is quite noteworthy that NO• and/or $ONOO^{-}$ directly upregulates production of IL-1β, TNF-α, IL-8, and hydrogen peroxide in macrophages. Nitrogen radicals also indirectly enhance cytokine induction of TNF-α [82–88]. This induction is mediated at the transcriptional level possibly through the induction of NFκB [82, 83, 86]. Lander et al. [83] suggest that NO•, through enhancement of GTPase activity and G protein mediated events, stimulates the translocation of NFκB to the nucleus. Nevertheless, in some cases NO• inhibits LPS-induced IL-1β and TNF-α in macrophages [87, 88]. In endothelial cells NO• inhibits NFκB translocation by stabilizing the complex of NFκB and its inhibitor by preventing degradation of the inhibitor [89, 90]. These cases illustrate the complexity of the effects of free radicals in signal-transducing events in the macrophage at different stages of activation and point to the danger in generalizing NO• effects on NFκB in all cells.

Other effects of NO • on the immune system

Is it possible that NO• protects against autoimmune disease? Might NO• regulate other aspects of the immune response in addition to cytokines or indirectly as the result of its effects on cytokines? Mutant mice in which the NOS-2 gene is defective, and which have been infected with *Leishmania major* have a significantly stronger Th1 type of immune response than wild-type mice [91], suggesting that NO• inhibits T cell responses, leading to delayed type hypersensitivity. NO• may inhibit T cell proliferation [92], either through suppression of IFN-γ [93] or induction of prostaglandin E_2 [94]. NO• induces apoptosis of thymocytes and may do so as well in the CNS [95]. Clearly the accessibility of NO• to intramolecular sites explains its diverse effects on ion channels, tyrosine kinases, phosphatases, and transcription factors, any of which may mechanistically account for alterations in the immune response (reviewed in [96]). NO• has also been shown to inhibit leukocyte adhesion and migration by its interference with CD11/CD18 (leukocyte functional antigen 1) expression [97]. NO• also downregulates MHCII expression in macrophages, thereby inhibiting antigen presentation [98]. In other words, early in the disease process in MS or EAE NO• might actually protect against autoimmune events initiated in the peripheral blood.

Evidence for NO • production in experimental models of MS and encephalitis

Although the potential role of NO• and NOS-2 in the pathogenesis of inflammatory disease has been appreciated since the late 1980s, it is only recently that significant progress has been made in studying the role of NO• in the pathogenesis of experimental animal models of MS and other neuroimmunological disorders. MacMicking et al. [99] first reported elevated spontaneous NO• and O_2^{-}• release ex vivo by both peripheral and CNS-derived neutrophils and mononuclear cells isolated from Lewis rats with acute guinea pig spinal cord homogenate-induced EAE. The release of both free radicals was augmented by incubation of these cells with encephalitogenic T cells, probably via the release of the proinflammatory cytokines TNF-α, IL-1β, and IL-2. An important aspect of this study was the observation that both peripheral and CNS-derived cells produced NO•, suggesting that the inflammatory cells responsible for mediating EAE in this model are likely to be activated prior to entering the CNS.

Koprowski et al. [100] used NOS-2-specific oligonucleotide primers and reverse-transcriptase polymerase chain reaction (RT-PCR) to evaluate NOS-2 induction in the brains of rodents with both encephalitic viral diseases and EAE. Intraocular injection of herpes simplex virus type 1 was associated with NOS-2 mRNA expression in all six mice with clinical signs of encephalitis 5 and 6 days p.i., although not all animals showed histological signs of inflammatory cell infiltrates. In rats infected with Borna disease virus (BDV) NOS-2 mRNA induction was observed to be highest on day 26 p.i., at a time when animals had severe neurological symptoms associ-

ated with perivascular necrosis. The correlation of NOS-2 mRNA induction and disease symptoms was not, however, absolute. Similarly equivocal results were obtained in mice treated with rabies virus, all seven of which exhibited severe clinical symptoms, but only three of seven NOS-2 mRNA induction. In guinea pig MBP-induced acute Lewis rat EAE NOS-2 mRNA induction was evident prior to (days 5 and 9), during (day 13), and after (day 19) clinical symptoms. We have obtained a very similar time course for NOS-2 mRNA induction in Lewis rat EAE (Garcia-Merino, Medberry, and Parkinson, unpublished observations).

To date no definitive analysis has been reported in any EAE model for the kinetics of NOS-2 mRNA induction and NOS-2 protein expression in conjunction with in situ hybridization and immunohistochemistry to identify NOS-2 mRNA and protein localization at the cellular level. Such a study has been reported for the encephalitic BDV model in Lewis rats and provides useful information [101]. The mRNA for NOS-2 was not present in brains of normal rats but was increasingly expressed at days 14, 17, and 21 postinfection. Maximal NOS-2 mRNA expression at day 21 occurred when neurological signs of seizures, convulsions, and tremors were evident. TNF-α mRNA expression, which would be predicted to precede NOS-2 mRNA expression, was maximal at day 17 and significantly decreased at day 21. In situ hybridization revealed that NOS-2 mRNA colocalized with BDV RNA in basolateral parts of the cortex and the hippocampus but not other infected brain areas. The macrophage marker ED-1 and NOS-2 antibody were used to show colocalization of NOS-2 with macrophage cells in perivascular regions of the hippocampus. Not all ED-1 positive cells, however, were NOS-2 positive.

The reactivity of NO• with heme and nonheme iron (Fe) centers to form electron paramagnetic resonance (EPR) detectable Fe-NO complexes is well known and formed the basis of an elegant study by Lin et al. [102] of endogenous NO• formation in MBP-specific T cell-mediated adoptive transfer EAE. Definitive EPR spectra of Fe-NO complexes of iron-sulfur proteins with a characteristic g=2.04 signal were observed in all ten spinal cord samples from female SJL/J mice with EAE. Notably, the size of the g=2.04 signal was higher in all mice with EAE (intensity index 1.8–5.0) than in controls (intensity index 0–1.4) and was correlated with clinical disease in all cases. No evidence for the formation of EPR-detectable Fe-NO complexes was detected in peripheral tissues such as spleen, liver, and blood of the affected animals. In contrast to acute EAE in Lewis rats, which typically exhibit an acute monophasic disease, the SJL/J EAE model in this study exhibited a chronic relapsing-remitting disease course with animals exhibiting clinical symptoms for prolonged periods after T cell transfer. The observation that EPR-detectable NO• adducts could be detected in spinal cords of animals with EAE at 14–75 days posttransfer strongly supports the contention that NO• is an important mediator of chronic inflammation. This is important when considering the potential role of NO• in the pathogenesis of MS, which is a chronic disease.

Taken together, the results of the above studies show that NOS-2 mRNA and protein (and presumably enzyme activity) are induced in rodent EAE models of MS and rodent models of virally mediated inflammatory encephalitis. The potential for prolonged, high-output NO• biosynthesis as a mediator of oligodendrocyte and neuronal cell death in these models is thus clear but asks the question as to how much NO• is actually produced in these models and is it sufficient to mediate cytotoxicity. A partial answer to this question has recently been provided by Hooper et al. [103] using a novel method for spin trapping NO• in vivo. The method involves infusion of animals with diethyldithiocarbamate and ferrous sulfate/sodium citrate [104] for 30 min in vivo to trap NO•, followed by rapid tissue isolation and freezing in liquid nitrogen prior to EPR analysis. In adoptive T cell transfer Lewis rat EAE large amounts of NO• (20–30 μM) were observed in the spinal cord on 4 and 5 days after T cell transfer, correlating with hind limb paralysis on day 4 and general paralysis on day 5. Although elevated NO• levels were detected in brain, they were substantially less than in spinal cord, consistent with the ascending course of this disease with the spinal cord as the primary site of lesion development. The same study examined BDV and rabies models to direct inflammation to the brain. In both viral diseases NO• production in the brain was highest at the time of onset of neurological symptoms: 10 μM on day 20 in BDV and 12–30 μM on days 5–7 in rabies.

The importance of these observations with NO• spin traps in vivo is threefold. First, they establish that CNS tissues in both viral and T cell mediated encephalitis are exposed for prolonged periods to very high levels of NO•, which is known to mediate cellular cytotoxicity in a number of in vitro model systems. If anything, the spin-trap technique used would grossly underestimate the amount of NO• produced in vivo. Second, they establish that high-output NO• synthesis localizes to the site of inflammatory disease, i.e., predominantly spinal cord in EAE and brain in rabies and BDV. Third, they confirm and extend the utility of a new technique to directly determine the efficacy of therapeutic agents directed at inhibiting NO• production in the CNS, such as NOS inhibitors or inhibitors of NOS-2 induction (see below).

NOS inhibitors in EAE

The studies described above provide a strong rationale for testing the potential efficacy of NOS inhibitors in treating EAE. The first reported study of an NOS inhibitor in EAE was by Cross et al. [105] using MBP-specific T cell adoptive transfer in SJL/J mice. Aminoguanidine, a fairly selective but weak inhibitor of NOS-2, was used for the study at high doses: 100 and 200 mg/kg s.c. or 400 mg/kg i.p. daily, with treatment starting on the day of T cell transfer. These doses and routes of administration of aminoguanidine were selected based on in vivo

inhibition of NOS-2 in a systemic model of LPS-induced endotoxemia in the same mouse strain. A substantially lower mean maximum clinical score was observed at 400 and 200 but not at 100 mg/kg in aminoguanidine-treated animals than in those receiving placebo. A delay in disease onset was observed only at 400 mg/kg. Histological analysis of spinal cords from EAE mice revealed a reduction in inflammation, demyelination, and axonal necrosis, which reached significance at the highest dose only. The potential for nonspecific effects of aminoguanidine on the immune system was tested by assessing the effect of aminoguanidine on proliferation of MBP-specific T cells in response to MBP or concanavalin A. Although no effect of aminoguanidine was observed on MBP-treated cells, an effect on concanavalin-treated cells was observed in one experiment.

We have observed very similar results with high-dose aminoguanidine treatment (200–400 mg/kg per day) in both MBP-peptide induced and T cell adoptive transfer EAE in PLJ/SJL F1 female mice. At both doses of aminoguanidine significant inhibition of disease incidence, maximal severity, duration, and cumulative score were observed and were associated with decreases in the number of inflammatory foci in both the meninges and parenchyma (Brenner, Parkinson, Perez, and Steinman, unpublished results).

NOS inhibitors have also been studied in Lewis rat EAE and experimental allergic neuritis [106]. Four L-arginine analog NOS inhibitors were tested: *N*-monomethyl-L-arginine (100 mg/kg p.o. daily), *N*-nitro-L-arginine (87.5 mg/kg p.o.), aminoguanidine (50 and 200 mg/kg i.p.) and *N*-nitro-L-arginine methyl ester (150 mg/kg i.p.). Ammonium acetate was used as a control. In experimental allergic neuritis a modest protective effect on disease score, compound muscle action potential, demyelination, and inflammation was seen for *N*-monomethyl-L-arginine only. *N*-Nitro-L-arginine methyl ester had a modest effect on clinical score only, and neither aminoguanidine nor *N*-nitro-L-arginine had any effect on any parameter studied. In EAE no beneficial effect of any compound was observed. Another EAE study in Lewis rats using *N*-nitro-L-arginine (125 mg/kg twice a day i.p.) and *N*-monomethyl-L-arginine (225 μg/kg once a day by intraventricular injection) showed a modest exacerbation of clinical score, suggesting a protective role for NO• in this model [107].

The disparity between the aminoguanidine results obtained in Lewis rat EAE and those in SJL/J mouse EAE is difficult to reconcile. The Lewis rat study did use lower doses and a different route of administration than the SJL/J study, and these differences may account in part for the disparity. An additional shortcoming of both these studies is that neither showed that the administered NOS inhibitor actually inhibited NOS-2 in situ, or that NO• production in the affected tissue was actually blocked. Access to these data and more information regarding the bioavailability of aminoguanidine and other standard L-arginine analog NOS inhibitors to the spinal cord of both rats and mice would help to rationalize these observations and provide direction for future research.

Future studies on the role NO• in the pathogenesis of EAE and other encephalitic diseases

The studies reviewed above clearly show that substantial progress has been made towards identifying NO• as a potential toxic mediator in inflammatory encephalitic diseases. However, much research is still required to consolidate the concept that NO• is central to disease pathogenesis, and that blocking NOS-2, either by direct enzyme inhibitors or by indirect antagonists of enzyme induction, would be an effective means for treatment.

To date there is still a lack of definitive data on the kinetics of NOS-2 induction in any EAE model and its correlation with disease onset/recovery and localization. Specifically, further studies are required to (a) define the precise kinetics and cellular localization of NOS-2 mRNA induction prior to, during, and after development of clinical signs; (b) test the correlation of the kinetics of NOS-2 mRNA induction with immunohistochemical localization of NOS-2 protein, enzyme activity, and NO• formation specifically due to NOS-2 and not other NOS isoforms; (c) and compare NOS-2 mRNA, protein, and enzyme activity in acute EAE models with monophasic disease versus chronic relapsing/remitting EAE.

The importance of analyzing the precise cellular localization of NOS-2 expression in these disease models cannot be stressed too highly, since this is likely to lead to a better understanding of the potential role of NO• in disease pathogenesis. The cell types that seem most relevant to the study of EAE are resident CNS microglia, blood-borne macrophages that enter the CNS, perivascular macrophages, T cells, and endothelial cells. With the exception of perivascular macrophages, all these cell types have been shown to have a cytokine-inducible NOS-2, at least in vitro. As summarized in Fig. 2, high-output NO• biosynthesis could play a number of roles in the pathogenesis of EAE. Activated resident microglia and blood-borne macrophages could contribute to primary demyelination through NO• mediated killing of oligodendrocytes. Due to their close apposition to the endothelium, induction of NOS-2 in astrocytes and/or perivascular macrophages could maintain or even exacerbate loss of blood-brain barrier function [108–109], an aspect of EAE which is not often studied. Endothelial cells could use endothelial (type 3) NOS, or NOS-2 as a compensatory mechanism to limit cellular traffic across the endothelium via down-regulation of adhesion molecules or chemokine release. An additional immunomodulatory role for NOS-2 could be local regulation of the inflammatory response in the CNS via NO• mediated apoptosis and/or necrosis of macrophages and encephalitogenic T cells.

With regard to the use of NOS inhibitors for treatment of EAE there is clearly a need for much better tools to perform these studies. The current generation of substrate-based NOS inhibitors, such as aminoguanidine, *N*-methyl-L-arginine, and *N*-nitro-L-arginine, appear to have outrun their utility for testing this concept. Their lack of both potency and clearly demonstrable CNS bioavailabili-

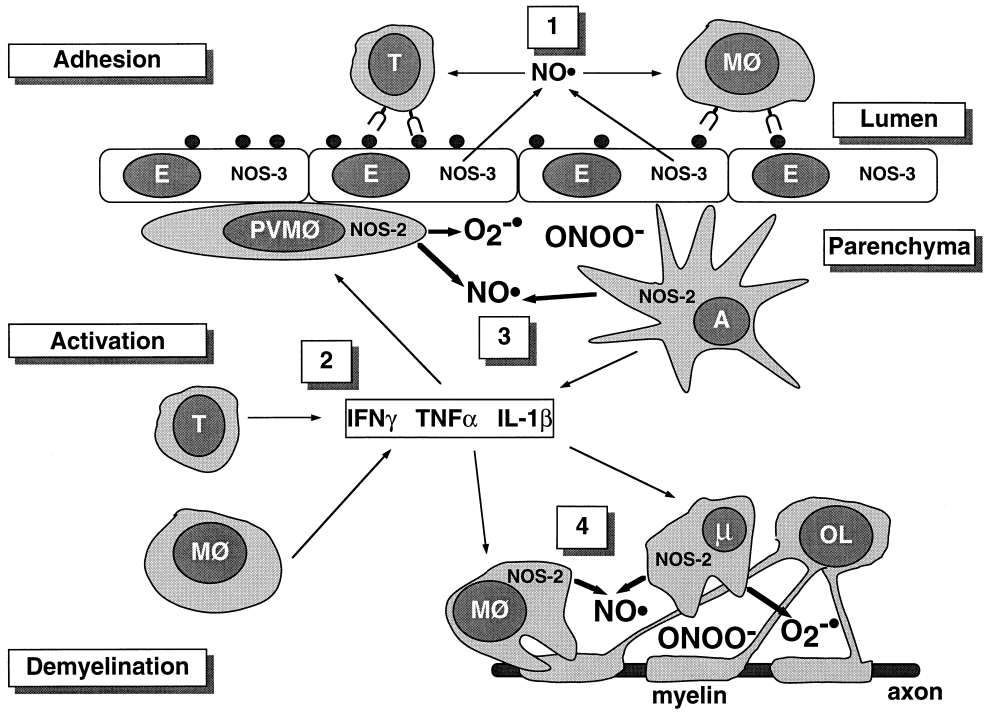

Fig. 2 The role of NO in the pathogenesis of MS and EAE. The schematic presentation shows three phases in the pathogenesis of human MS and rodent EAE: adhesion, activation, and demyelination. Extravasation of encephalitic T cells (*T*) and circulating macrophages (*MØ*) from the vessel lumen to the CNS parenchyma is mediated by chemotactic stimuli (not shown) and the expression of adhesion molecules (*solid circles*) on brain endothelial cells (*E*). Release of small amounts of nitric oxide (*NO•*) by endothelial nitric oxide synthase (*NOS-3*) may act locally as a homeostatic mediator (*1*) to downregulate adhesion molecule expression and T cell or macrophage activation status. In the parenchyma the release of the proinflammatory cytokines IFN-γ, TNF-α, and IL-1β (*2*) results in activation of perivascular macrophages (*PVMØ*), astrocytes (*A*), macrophages and microglia (*m*), including expression of inducible NOS (*NOS-2*). High-output NO• and superoxide (O_2^{-}•) release, which can react to form the powerful oxidant peroxynitrite ($ONOO^{-}$), are proposed to mediate nonspecific tissue damage at two sites. Oxidants released by perivascular macrophages and astrocytes may exacerbate loss of blood-brain barrier function (*3*) by promoting endothelial dysfunction. Oxidants released by phagocytic macrophages and microglia may also contribute to primary demyelination (*4*) via nonspecific damage to the myelin sheath of axons and promoting direct oligodendrocyte (*OL*) cell death. Potential roles for NOS-2 in downregulating the inflammatory response are discussed in the text

ty appears severely to limit their utility for EAE treatment. Their known lack of selectivity renders any efficacy results achieved with them in EAE highly suspect in the absence of any clear pharmacological evidence that their administration leads to NOS-2 inhibition without inhibition of neuronal (type 1) NOS or endothelial (type 3) NOS activity. Although an intense effort has been made, at the current time there does not appear to have been a significant advance in the design of potent, selective NOS-2 inhibitors with sufficient pharmacological profiling in the CNS to achieve a breakthrough in this area.

Given the limitations of the current generation of NOS-2 inhibitors, what can be done to further understand the role of NOS-2 in EAE pathogenesis? Fortunately, transgenic NOS-$2^{-/-}$ (knockout) mice have recently been generated [35, 91] and are currently being backcrossed with mouse strains susceptible to EAE and other spontaneous autoimmune and inflammatory diseases. It is anticipated that data on the effect of the NOS-$2^{-/-}$ phenotype on EAE will be available in the not too distant future. Alternatively, as reviewed above, a number of approaches to inhibiting inflammation have been or are being tested for treatment of EAE. These include blocking proinflammatory cytokines such as TNF-α or IL-1β by using either neutralizing antibodies, receptor antagonists, or inhibitors of synthesis (e.g., phosphodiesterase inhibitors), blockade of chemokines such as macrophage inflammatory protein 1α [109] and treatment with anti-inflammatory cytokines such as IL-10, TGF-β, and IL-13. Although evidence for downregulation of NOS-2 using these approaches exists in vitro, the effects of these treatment strategies on NOS-2 induction in vivo have yet to be characterized. Is the efficacy of these agents correlated with NOS-2 blockade, or do they work without affecting NOS-2 induction and NO• production? Answers to these questions are clearly necessary to define the role of NOS-2 and NO• as a central pathological mechanism in EAE.

Evidence for NO• induced damage of oligodendrocytes in vitro

In culture the myelin-producing cell, the oligodendrocyte, is vulnerable to toxicities mediated by complement, antibodies, cytokines, oxygen free radicals, and nitric oxide produced by macrophages [28, 29, 33, 111]. Studies performed in this laboratory have demonstrated an IFN-γ

induced, NO• dependent microglia cell-mediated cytotoxicity of oligodendrocytes [28]. Ameboid rodent microglial cells, after treatment with phorbol myristate acetate, LPS, and/or IFN-γ, produce micromolar concentrations of NO• within 24 h. NO• production, as well as oligodendrocyte lysis, is inhibited by NOS inhibitors and anti-TNF-α antibodies, thus suggesting that NO• is the mechanism of macrophage/microglial cell killing of oligodendrocytes. These in vitro results suggest a direct role for NO• in oligodendrocyte cell loss and primary demyelination in EAE and MS in vivo.

We have also examined the differential effects of nitric oxide on primary glial cells in vitro [112]. Cultures enriched for microglia, astrocytes, or oligodendrocytes were treated with S-nitroso-*N*-acetyl D,L-penicillamine, an NO• releasing chemical. There was a significant decrease in the function of the ferrosulfur-containing mitochondrial enzyme, succinate dehydrogenase, in oligodendrocytes and astrocytes treated with S-nitroso-*N*-acetyl D,L-penicillamine, whereas microglia were unaffected. In addition, morphological changes and single-stranded DNA breaks occurred in oligodendrocytes but not in astrocytes and microglia. Oligodendrocytes were also less easily rescued from the toxic effects of NO• by oxyhemoglobin than were astrocytes. A subpopulation of oligodendrocytes were killed by NO• via a necrotic, non-apoptotic mechanism [113]. These findings strongly suggest that the myelin-producing cell is more sensitive to NO• than the other two glial cell types.

Several laboratories have examined in vitro production of NO• by human blood macrophages and microglia. While some reports have failed to detect NO• synthesis in macrophages stimulated with LPS and cytokines such as IFN-γ or IL-1β alone, other studies have demonstrated that human macrophages are capable of producing micromolar concentrations of NO• upon cross-linking of cell surface molecules other than cytokine receptors [114]. Interestingly, human and rodent macrophages differ not only in stimulus required for NO• induction but also the time required to detect NO• in cell culture supernatants. IFN-γ, IL-1β, and TNF-α induce production of micromolar concentrations of NO• by rodent macrophages and do so within 24 h. Human macrophages require 3–5 days after stimulation before NO• is detectable [114].

Human glial cells have been reported to produce NO• but can be distinguished from human blood macrophages in that LPS can stimulate NO• production in malignant astrocytoma cell lines. The best inducer of NO• production in normal human fetal astrocytes is IL-1β alone or in combination with other cytokines such as IFN-γ [115]. LPS is not a good inducer in these cells. Colasanti et al. [115] have reported induction of NOS-2 mRNA by LPS and IFN-γ in human microglial cells as determined by using RT-PCR and Southern analysis. We have also demonstrated the production of NOS-2 and NO• in cultures of fetal human microglia and astrocytes. Glia from adult brain tissue also produce NO• but not in response to cytokines alone, suggesting differential developmental regulation of NOS-2 ([116, 117] and manuscript in preparation).

Evidence for involvement of NO• in the pathology of MS

Direct and indirect methods for the presence of NO• and the induction of NOS-2 have recently been reported in MS patients. Analysis of CSF from MS patients has shown increased levels of nitrate and nitrite compared with normal controls [118]. The same study reported increased levels of neopterin, a precursor of the NOS-2 enzyme cofactor tetrahydrobiopterin, in CSF from MS patients. The reaction of NO• with O_2^{-}• forms peroxynitrite, $ONOO^{-}$, a strong *trans*-nitrosating agent capable of nitrosating susceptible protein thiols, such as cysteine. This chemical reaction may result in formation of nitroso-amino acids, such as nitrosocysteine, potentially making them immunogenic. It is therefore of interest that significantly elevated levels of circulating IgM antibodies to a conjugated *S*-nitroso-cysteine epitope have been detected in MS sera compared with sera from patients affected with other neurological and autoimmune diseases [119]. Both of these studies provide indirect evidence for the chronic production of elevated NO• levels in MS.

Work from three different laboratories has shown evidence for the induction of NOS-2 in the brains of MS patients. Brosnan et al. [120] demonstrated intense NADPH diaphorase histochemical staining colocalized with glial fibrillary acidic protein staining of astrocytes, as well as with TNF-α and IL-1β, in acute MS lesions. Bo et al. [121] have also demonstrated NADPH diaphorase histochemical staining in the brains of MS patients. According to the morphology of the cell and glial fibrillary acidic protein immunoreactivity, they too concluded that the majority of NO• producing cells were reactive astrocytes. They also showed by semiquantitative RT-PCR that the levels of NOS-2 mRNA were markedly higher in MS brains than in normal brains. Due to the lack of specificity of the NADPH diaphorase stain [122], however, neither of these studies unequivocally elucidated the cellular source of NOS-2 and hence NO• in MS tissue. Quite recently, Bagasra et al. [123] have demonstrated more directly the source of NOS-2 mRNA and functionality of NOS-2 enzyme in MS tissue. The mRNA for NOS-2 was detectable in all the brains examined from patients with MS and other neurological diseases but in none of the control brains. In situ RT-PCR experiments also demonstrated the presence of NOS-2 mRNA in the cytoplasm of the cells which expressed the ligand recognized by the *Ricinus communis* agglutinin 1, a marker for macrophage/microglia. In addition, the presence of nitrosylated proteins in MS lesions was assessed with an anti-nitrotyrosine antibody and found to colocalize with NOS-2 mRNA and protein in these macrophage/microglial cells. These observations are very significant since they indicate not only the presence of active NOS-2 enzyme in macrophage/microglial cells of the MS lesion but also concomitant O_2^{-}• production, the formation of $ONOO^{-}$, and subsequent protein nitrosation.

Future studies on the role of NO• in the pathogenesis of MS

These early studies with human brain tissue and fluids clearly point to NO• as a potential toxic mediator in the inflammatory, demyelinating pathology of MS. The histopathological studies in human MS brains are in general agreement with those in rodent EAE, with mounting evidence for the formation of NO• by both astrocytes and macrophages/microglia. The latter cells appear to be the major, but not exclusive, site of $ONOO^-$ formation, most likely through interaction of NO• produced by NOS-2 with O_2^{-}• produced by the respiratory burst NADPH-oxidase of these cells. The local production of NO•, O_2^{-}•, $ONOO^-$, and other oxidizing free radicals via activation of both these enzyme systems in macrophages/microglia may contribute directly to oligodendrocyte destruction and primary demyelination in vivo. This hypothesis is consistent with an effector function for activated, phagocytic macrophages/microglia in MS pathology and an "innocent bystander" role for the oligodendrocyte. The potential contribution of astrocyte NOS-2 to MS or even EAE pathology is far less clear, as is any immunomodulatory role on immune cell function.

In order to better understand the role of NO• in the pathogenesis of MS further studies are clearly needed. We would suggest: (a) a precise analysis of NOS-2 expression and cellular localization in the brain with respect to the state of the lesion (active vs. chronic) and also clinical disease stage (i.e., chronic vs. relapsing-remitting) and (b) a more complete analysis of CSF or other body fluids of MS patients for NO• metabolites such as nitrite, nitrate, 3-nitrotyrosine, or nitrosylated proteins and correlation of these with disease stage (chronic vs. relapsing-remitting).

The studies suggested above will shed further light on the role of NO• in the pathogenesis of MS and will likely provide further support for the development of NOS-2 inhibitors as potential MS therapeutics. The analytical techniques for NO• related metabolites are constantly being enhanced. These tools are likely to be of value for clinical research neurologists with an interest in determining the effect of current and novel MS treatment regimens on the NOS-2 pathway in vivo and the correlation of NOS-2 blockade, if any, with clinical outcome.

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