Nitric Oxide and Multiple Sclerosis

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Nitric oxide (NO) is a free radical signaling molecule with remarkably complex biochemistry. Its involvement in multiple sclerosis (MS) had been postulated soon after the discovery of the critical role NO plays in inflammation. However, the extent of NO's contribution to MS is not yet understood, party due to the often opposing roles that NO can play in cellular processes. This review briefly covers new developments in the area of NO that may be relevant to MS. It also describes recent progress in understanding the role of NO in MS, new potential targets of the action of NO in the cell, and prospects for NO–based therapies.

Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS). It is characterized by a chronic inflammatory process resulting in oligodendrocyte and axonal damage and eventual neuronal loss. Nitric oxide (NO), a versatile signaling molecule, was implicated in the inflammatory process soon after its discovery as an endogenously produced signaling molecule, and the potential contribution of NO to the development of MS has been extensively tested in humans and in animal models.

Abundant evidence 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. Much of this evidence is correlative, although a number of reports more directly test the involvement of NO in the disease. However, there are still many ambiguities in the picture of how NO contributes to MS, and we are still far from developing an NO-based therapy for preventing, stopping, or reversing the damage inflicted by MS. Much of the difficulty is related to the complex manifestation of the disorder; however, much of it is also due to the multiplicity of functions that NO subserves in the organism and to the unusually complex biochemistry of NO in normal and inflamed tissue. Almost two decades after the discovery of endogenously produced NO, we are still learning about the basic mechanisms regulating its production, chemistry, storage, and transport; even the range of its cellular targets is still being charted.

In this review, we describe recent developments in the field of NO and MS research that may have direct implications for our understanding of MS-associated pathology and for designing NO-based therapies for MS (for a comprehensive review of the link between NO and MS see Smith and Lassmann [1••]; also see Parkinson *et al.* [2], Smith *et al.* [3], and Willenborg *et al.* [4] for reviews). However, we first touch upon some recent developments relating to the physiology of NO production in the organism.

Nitric Oxide Production and Targets

Although NO was discovered as a signaling molecule regulating vasodilation, neuronal function, and immune response under normal conditions, it also emerged as a key player in several pathophysiologic processes, ranging from hypertension and diabetes to neurodegeneration and cancer [5–7]. Enzymatic synthesis of NO from arginine is mediated by NO synthases (NOS), which in mammals are encoded by three genes corresponding to neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) isoforms [8].

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 (micromolar vs nanomolar local concentration) [8]. iNOS expression is mainly controlled at the level of transcription and can be induced by an appropriate combination of cytokines or by endotoxins in almost every cell type. The involvement of iNOS in inflammation and the immune response has been thoroughly documented [9] and is directly related to the high levels of NO produced by the enzyme after transcriptional induction.

The chemistry and biochemistry of NO are remarkably complex. Given that various oxides of nitrogen can also react with reactive oxygen species (ROS), the list of the reactive nitrogen intermediates (RNI) and ROS with demonstrated biologic activities continues to grow [7,10,11]. This expanding catalog of derivatives of NO is paralleled by an expanding list of proven and potential cellular targets of NO. Reaction with the heme iron of the

soluble guanylate cyclase remains the best characterized example of NO action. Through generation of cyclic GMP, this pathway mediates much of the physiologic response to NO and underlies the action of several therapeutic drugs. However, it is increasingly clear that other NO-dependent modifications of cellular components are also employed for transduction of the NO signal in cells and tissues.

Nitrosylation of the sulfhydryl groups in proteins (*ie*, generating S-nitrosothiol [RSNO] compounds) has been proposed as a major regulatory modification, and there is an impressive list of examples where S-nitrosylation of cysteines acts to control protein activity [12] or stability [12–14]. It has recently been proposed that the true extent of the nitrosylation of the peptidyl sulfur in proteins is currently underestimated. The survey of various tissues estimates that the concentration of RSNO-containing proteins is in the nanomolar range and is comparable with the concentration of NO-heme–containing proteins in those tissues [15••,16]. Remarkably, the same survey presents a challenging indication that another modification, nitrosation of amines (generating N-nitrosamines [RNNO] compounds) may be as widespread in proteins as S-nitrosylation. Furthermore, N-nitrosylation is dependent on the activity of NOS and is dynamically regulated (*eg*, by hypoxia). The importance of this discovery relates to the fact that nitrosamines have been considered only as potential carcinogens and that the physiologic role and relevance of N-nitrosylation of proteins is virtually unexplored.

Another widely studied NO-induced protein modification reflects the reaction of NO with the superoxide radical O_2^-, which generates highly reactive peroxynitrite ($ONOO^-$). The production of peroxynitrite leads to the nitration of tyrosine residues in proteins; the appearance of nitrotyrosines is considered a hallmark of pathologic changes caused by nitrosative stress in cells and tissues. Although usually employed as a surrogate marker of inflammation, tissue degradation, or a drastic shift of the redox balance, tyrosine nitration may itself serve as a regulatory signal, a possibility that has not yet been sufficiently investigated.

An exciting new discovery comes from the re-evaluation of the biologic activity of lipids modified by nitrogen oxides. Baker *et al*. [17••] have demonstrated the presence of nitroderivatives of linoleic acid in healthy individuals and found that the nitrated lipids are present in the plasma in amounts surpassing any of the other bioactive oxides of nitrogen (*eg*, more than 100 times the amount of RSNO compounds). Thus, the nitrated lipids have the potential to serve as a depot of nitrogen oxides (also note that membranes may serve to increase the local concentration of NO by acting as a "molecular lens" [18]). Importantly, the nitrated lipids have demonstrated a profound anti-inflammatory activity (*eg*, inhibiting platelet and neutrophil activation), possibly due to their ability to activate peroxisome proliferator activated receptor γ [19]. Thus, nitrated lipids may represent a new class of anti-inflammatory compounds. Furthermore, this finding is an example of the convergence of anti-inflammatory and proinflammatory (oxidized lipid-dependent) signaling pathways [17••]. Note that a range of other lipids can potentially be nitrated under acidic conditions (*eg*, in the gastric system or upon acidification of endosomes), and thus can be included into the metabolism of the biologically relevant nitrogen oxides. It remains to be determined whether an enzymatic system participates in nitration/denitration of lipids, how much of the action of nitrated lipids is due to their release of NO, and what is the range of signaling pathways activated by nitrated lipids.

The arginine-based enzymatic production of NO by NO synthases is clearly an important source of biologically active NO; remarkably, however, it is not the sole source of NO in humans. Nonenzymatic production of NO and other RNIs is mediated by nitrate-reducing commensal bacteria and occurs in the acidic environment of the stomach (and probably skin, oral cavity, and even acidified cell compartments). Under this scenario, ingested nitrates are absorbed into the bloodstream and, although most of them are excreted in urine, a fraction accumulates in the saliva and sweat. The oral cavity and skin are heavily colonized by commensal bacteria, many of which are capable of effectively reducing nitrates to nitrites. The entero-salivary circuit should not be underestimated because the salivary gland takes up nitrates very effectively: up to 25% of the plasma nitrate is recovered in the saliva and the concentration of nitrates in saliva (up to 1 mM) is more than 10 times higher than that in plasma. The crucial transformation occurs when the salivary nitrites enter the highly acidic environment of the stomach and generate nitrous acid, dinitrogen trioxide, nitrogen dioxide, and NO (see Lundberg *et al*. [20•] for a comprehensive review describing the entero-salivary circulation of nitrates and production of NO in humans). Highly reactive RNI species have antimicrobial activity and serve to kill enteric pathogens. These RNI may also be relevant for inflammation (*eg*, in MS) because they can also modify lipids and thus generate an important reservoir of anti-inflammatory activity.

Together, these recent advances in the NO field serve as a reminder that NO metabolites may have opposing activities in inflamed tissue, that NO-dependent modifications of biomolecules generate a wide variety of active compounds, and that dietary sources of nitrate may, potentially, affect NO-dependent processes in the organism.

Nitric Oxide Synthase Expression and Nitric Oxide Production in Multiple Sclerosis

Because the iNOS isoform is the high-output producer of NO and because its role in inflammation had been well established, the main focus of studies of the potential role of NO in MS was, early on, directed towards this isoform and towards the signs of massive NO production. Indeed, there is still no compelling evidence for the contribution of

nNOS or eNOS activity to the disease (although this possibility may not yet have been sufficiently investigated). The possible association between iNOS and MS has been addressed in several genetic linkage studies. Two broad studies focusing on polymorphisms in the promoter region of the iNOS gene failed to reveal any direct association between variants of the iNOS gene and susceptibility to MS [21,22]; a similar analysis did not find an association between MS and the neuronal isoform of NOS [23]. However, a recent comprehensive analysis of single nucleotide polymorphisms in 34 genes related to inflammatory pathways revealed significant association between MS susceptibility and changes in the iNOS gene (a silent substitution in exon 10 or differences in the promoter region) [24], thus providing the first genetic evidence that variations within the iNOS gene may contribute to disease susceptibility.

Although iNOS RNA and protein are present at very low levels in the CNS under normal conditions, their presence is well documented both in the CNS of patients with MS and animals with experimental autoimmune encephalomyelitis (EAE), a well-established model of MS. In the EAE model, the level of expression of iNOS correlates with the severity of clinical signs [1••], and a drug combination that can reverse EAE also reduces the NO production in the animals [25•]. In MS patients, strong iNOS immunoreactivity has been found in active lesions, and the signal is reported to be weaker in less inflamed lesions [1••]. More recent reports confirm these observations. iNOS was detected in postmortem magnetic resonance imaging—guided biopsies from patients with definite MS, both in the active lesions and in the white and gray matter regions that appeared normal, with most of the immunoreactivity detected in the reactive astrocytes [26]. Expression of iNOS was also observed in active plaques from MS patients displaying both acute demyelination and active inflammation, with most of the signal present in the ependymal cells in periventricular lesions, in astrocytes, and in macrophages/microglial cells [27].

Most of the endogenously produced NO is ultimately converted to nitrates and nitrites, which accumulate in the plasma and are excreted in the urine, saliva, and sweat. The amount of secreted nitrates/nitrites and their concentration in the plasma are increased during systemic inflammation (the link between inflammation and nitrate/nitrite production was one of the key observations leading to the discovery of NOS). Thus, there had been a long history of attempts to correlate the course of MS with changes in nitrate concentration in plasma, cerebrospinal fluid (CSF), and urine. Indeed, there have been several reports describing an increase in the nitrate and nitrite concentration in the CSF of patients with MS. Other reports, however, were unable to find such an increase [1••]. In new studies on MS patients, a positive correlation between the levels of nitrates/nitrites and clinical disease activity has been found [28,29]. Furthermore, in a follow-up study of a cohort of MS patients divided into groups with relapsing-remitting, primary progressive, and secondary progressive MS, it was found that the nitrate/nitrite levels were higher in the CSF of patients with disability progression than in those who were clinically more stable [30]. Thus, recent reports support the idea that the increased nitrate/nitrite concentration in the CSF correlates with disease progression and may serve as a surrogate marker for disease activity. The reports trying to correlate disease progression with the concentration of nitrates/nitrites in the plasma produce less convincing results [1••,28], perhaps because of the dilution of the CSF nitrates in serum, the effects of diet, or the potential peripheral inflammation.

Nitric Oxide-mediated Damage to Oligodendrocytes and Neurons

When exposed to reactive nitrogenous species (RNS), oligodendrocytes are more susceptible to NO-mediated toxicity than astrocytes or microglia [31]. Most of the destructive action of NO is likely due to the formation of peroxynitrite through reaction with superoxide. Among its other activities, peroxynitrite leads to nitration of tyrosine residues in proteins; conveniently, this NO-related protein modification can be readily visualized by means of antibodies against nitrotyrosine. Thus, the presence of nitrotyrosine is often monitored as a footprint of the pathophysiologic activity of NO in the inflamed tissue. Indeed, nitrotyrosine labeling was detected in hypertrophic astrocytes and activated microglia in MS lesions [27,32–35]. The causative role of peroxynitrite in oligodendrocytic damage is supported by findings that a peroxynitrite scavenger, uric acid, can inhibit demyelination and axonal damage (note that gout and MS are mutually exclusive conditions) [36,37]. Peroxynitrite-mediated damage to oligodendrocytes seems to be independent of poly(ADP-ribose) polymerase (PARP) activation [38]. This finding stands in contrast to the important role that PARP activation plays in neuronal toxicity, perhaps indicating that alternate cytotoxicity pathways are activated by peroxynitrite in oligodendrocytes. The cytotoxic effect of peroxynitrite on oligodendrocytes can be attenuated by pretreatment with 17β-estradiol [39], but again, the signaling pathways involved in protection are not clear.

Besides activating the general mechanisms of cell death, peroxynitrite may damage the myelin sheath through lipid peroxidation, which affects both the lipid and, indirectly, the protein components of the membrane. Intriguingly, NO may also affect the structure of the myelin sheath more directly (*ie*, not in a peroxynitrite-dependent manner). Exposure to NO donors causes myelin decompaction, accompanied by S-nitrosylation of a cysteine-rich proteolipid protein (PLP) [40•]. This abundant component of the CNS myelin is important for the structural integrity of the sheath, and nitrosylation of the cysteine residues may alter its structure and

compromise its function. Interestingly, peripheral nervous system myelin, whose stability depends on proteins other than PLP, does not show decompaction upon incubation with the NO donors. In agreement with these in vitro observations, elevated levels of nitrosothiols were found in the CSF of the patients with active MS [41], and an increase in the levels of anti–S-nitrosocysteine antibodies was observed in MS patients [42•] and animals with EAE [43]. In the EAE animals, the serum levels of anti–S-nitrosocysteine antibody peaked 1 week before the onset of clinical signs and the antibody titer correlated with the extent of subsequent demyelination [43]. In patients with relapsing-remitting MS, elevated levels of anti–S-nitrosocysteine antibodies were found at times of relapse, whereas the levels were normal in patients in remission [42•]. The antibody titer was also elevated during acute MS attacks and in progressive disease. Together, these studies suggest that cysteine modifications by NO may play a crucial role in the damage to the myelin sheath induced by inflammation or nitrosative stress; they further indicate that the appearance of anti-S-nitrosocysteine antibodies in the blood may be used as a marker of clinical activity [42•]. It will be important to follow these observations with structural studies of the modified PLP and other protein components of the myelin sheath after exposure to NO and peroxynitrite.

Nitric oxide may also affect the integrity and survival of oligodendrocytes by interfering with the glutamate release and uptake. NO can augment glutamate release [5,6], and this may potentially lead to glutamate receptor overactivation in oligodendrocytes and neurons and may also lead to excitotoxic cell damage and death. Oligodendrocytes are particularly vulnerable to the elevated concentrations of glutamate [44–46]. Given that expression of the main glutamate transporters in oligodendrocytes is suppressed both in MS and in EAE [47], it is plausible that NO helps to perpetuate the glutamate-mediated damage to oligodendrocytes and neurons during inflammation by both increasing the release of glutamate and suppressing its reuptake.

Nitric oxide–mediated glutamate excitotoxicity may be particularly damaging to the nervous system when combined with hypoxic conditions. NO released from inflammatory-activated glial cells leads to a 10-fold increase in the number of apoptotic and necrotic neurons when combined with hypoxia [48•]. Importantly, this synergism between NO and hypoxia is mediated by glutamate and can be prevented by an *N*-methyl-D-aspartate–receptor blocker. Thus, a combination of inflammatory and hypoxic condition may sensitize the neurons, and, potentially, oligodendrocytes, to NO- and glutamate-mediated damage. It is of note that in a subset of MS patients, the pattern of lesions shows remarkable similarity to the alterations found after white matter stroke, leading to the suggestion that a hypoxia-like injury is an essential component of the demyelinating inflammatory lesions [49,50].

The mechanisms of NO- and glutamate-mediated injury to the CNS may include interactions with zinc-activated pathways, as has been recently demonstrated for cortical neurons [51•]. In this model, overstimulation of glutamate receptors and excessive influx of calcium augments NO production and generation of peroxynitrite. This liberates zinc from intracellular stores; free zinc leads to mitochondrial dysfunction, including respiratory block, release of cytochrome c, activation of the Apaf-1/caspase apoptosis pathway, and further ROS production. Increased production of superoxide, in turn, results in even larger levels of peroxynitrite, which, through activation of the p38 mitogen-activated protein kinase, leads to potassium efflux and cell shrinkage, thus causing further progression of the cell death program. A similar molecular cascade may underlie the NO- and glutamate-mediated injury to oligodendrocytes, and this idea should be explored in cultured cells or in the EAE model.

Nitric Oxide-based Therapy

Overall, several lines of research present compelling evidence that NO has an important role in MS. However, attempts to achieve clinical gain by blocking NOS production have had limited success and, in some cases, led to contradictory results [1••,4]. Treatment of EAE animals with NOS inhibitors has been reported to have both beneficial and deleterious effects, and has even made strains that are normally resistant to EAE susceptible to the disease [1••,4,52•,53]. Furthermore, deletion of the iNOS gene, rather unexpectedly, increased the severity of EAE [52•]. The most probable explanation of this diverse array of results is related to the immunomodulatory activity of NO, which is compromised in the iNOS knockout animals or after exposure to NOS inhibitors. The beneficial effect of NO may be related to its ability to inhibit antigen presentation, T-cell proliferation, and recruitment of T cells and macrophages into the lesion; furthermore, it may be related to the anti-inflammatory activity of nitrated lipids [17••]. Indeed, the iNOS knockout mice with EAE show significantly increased proliferation of spleen and lymph node cells and increased production of T-helper cytokines [35], supporting the notion that immunomodulatory effects of NO contribute to the resistance to the EAE. Furthermore, the overall effect of NO may be related to the disease phase, because the outcome of exposure to NOS inhibitors seems to be highly dependent on the timing of treatment [54–56]. Together, the results from the experiments with genetically or pharmacologically suppressed NO production indicate that it is important to further dissect the signaling cascades activated by NO to be able to block its deleterious activities while allowing its beneficial effects. In particular, it will be important to explore the possibility of reducing the production or action of peroxynitrite (*eg*, using superoxide or peroxynitrite scavengers) without compromising the anti-inflammatory branch of the NO signaling pathways.

Nitric Oxide and Stem Cell Therapy

Although the basic causes of MS remain unknown, there is a range of approved therapies that provide statistically proven benefits by slowing down the course of the disease. However, there are no treatments to restore the loss of neurologic function when the demyelination becomes irreversible or when the axon or neuron is lost to the disease. Although EAE for restoration often undergoes spontaneous reversion and responds to prospective therapies, there is only anecdotal evidence of neurologic function in MS patients. Meanwhile, the recovery of neurologic damage is, of course, as desirable as the prevention of progression. The action of NO may also be relevant for a therapeutic strategy that is now actively considered: the use of exogenous, or recruitment of endogenous, stem/progenitor cells for the treatment of MS [57•]. The aspect of NO function that may be important for such approaches relates to its activity as a negative regulator of cell division in the adult CNS. NO and nNOS emerge as important contributors to the control of adult neurogenesis, such that exposure to pharmacologic inhibitors of NO production or genetic inactivation of nNOS results in an increased production of new neurons in the neurogenic areas of the adult brain [58–61]. Moreover, suppression of NOS activity affects the production of progenitor cells, including those that can give rise to oligodendrocytes (Packer, Encinas, and Enikolopov, Unpublished data). It will be important to explore the potential of NOS inhibitors or NO scavengers to help recruit progenitor cells in the CNS, in order to direct them towards oligodendrocytic differentiation, to instruct transplanted stem/progenitor cells to survive and acquire the fate of cells damaged by MS, and, even more challenging, to relocate to sites of damage.

Conclusions

Nitric oxide has a multifaceted role in MS and EAE. Its action may have both positive and negative effects on the development of the disease. It will be important to dissect the molecular mechanisms of the action of NO in the CNS to be able to retain the advantageous aspects of NO activity while blocking the detrimental aspects (*eg*, by only reducing the levels of peroxynitrite). Recent discoveries highlight the need to investigate the protein targets of the NO action in cells; likewise, the discovery of the endogenous nitrated lipids with anti-inflammatory activity opens an unexplored signaling realm with great potential for therapy. Furthermore, the possibility of nonenzymatic production of RNI may renew interest in the dietary contribution to the control of the disorder. Finally, the role of NO as a regulator of cell proliferation in the adult CNS should be explored in relation to prospects for stem cell therapy.

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