The Protective Role of Nitric Oxide in a Neurotoxicant-Induced Demyelinating Model

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Heather A. Arnett, Ron P. Hellendall, Glenn K. Matsushima, Kinuko Suzuki, Victor E. Laubach, Paula Sherman, and Jenny P.-Y. Ting

Demyelination is often associated with acute inflammatory events involving the recruitment-activation of microglia/macrophage, astrocytes, and leukocytes. The ultimate role of inflammatory products in demyelinating disease and in the survival of oligodendrocytes, the myelin forming cells, is unresolved. The current study examines the role of inducible NO synthase (iNOS)-derived NO in a neurotoxicant-induced model of demyelination. NO levels were greatly elevated in the midline corpus callosum during demyelination in genetically intact C57BL/6 mice, and this NO was due solely to the induction of iNOS, as the correlates of NO were not found in mice lacking iNOS. C57BL/6 mice lacking iNOS exhibited more demyelination, but did not display an increased overall cellularity in the corpus callosum, attributable to an unimpeded microglia/macrophage presence. An enhanced course of pathology was noted in mice lacking iNOS. This was associated with a greater depletion of mature oligodendrocytes, most likely due to apoptosis of oligodendrocytes. Microglia and astrocytes did not undergo apoptosis during treatment. Our results suggest a moderately protective role for NO during acute inflammation-association demyelination. The Journal of Immunology, 2002, 168: 427–433.

Glia lial cells in the CNS, particularly the microglia, contribute greatly to inflammatory responses in the brain, also known as neuroinflammation. This process of neuroinflammation is prevalent in a large number of CNS pathologies, such as Alzheimer’s disease, Huntington’s disease, trauma, AIDS dementia, amyotrophic lateral sclerosis, and multiple sclerosis (MS). These diseases include those with an immunologic etiology and those without one. The role of neuroinflammation in MS, which is generally considered an immune-mediated disease, has been studied extensively, primarily through the use of experimental autoimmune encephalomyelitis (EAE), an animal model for MS. However, the role of inflammatory molecules in other demyelinating conditions, specifically those with a less prominent immunologic component, is less well known. This is clearly an important issue, and it may greatly influence the treatment protocol for demyelinating diseases as a group. The focus of this study is to reveal the role of one potent inflammation-associated molecule, inducible NO synthase (iNOS), in demyelinating diseases without an obvious immunologic etiology.

iNOS, through the production of NO, is involved in many physiological mechanisms, and the number of its potential targets continues to expand. NO plays a critical role in brain function in general and is believed to have an important role in numerous neuropathological processes, including trauma, AIDS dementia, Alzheimer’s disease, and cerebral ischemia (1–6). In vitro exposure of neurons, astrocytes, or oligodendrocytes to high levels of NO can lead to significant cell death (7–10). These studies point to a potential role for NO in CNS pathology; however, direct evidence for its role in neuropathology is not well established.

MS is characterized by an immune response against components of myelin, resulting in the damage or loss of this critical element of neuronal function (11, 12). During the active stages of this response, infiltrating cells as well as endogenous activated microglia and astrocytes release multiple factors that may both exacerbate the disease and protect intact tissue from further damage. In MS, and in animal models of this disease, the production of free radicals, cytokines, and other pro- and anti-inflammatory molecules is correlated with specific states of disease progression (12). Strong circumstantial evidence supports the involvement of iNOS and NO in the disease. Induction of iNOS, NO, and NO-related byproducts has been found in MS (2, 13–16). Elevated levels of these molecules have also been found in EAE, a widely studied model for MS, and the level of this induction correlates with disease severity and cellular infiltrate (17–22). However, disruption of NO production in the EAE model has provided protection in some studies (23–28) but exacerbated the pathology in other experiments (29–32). Clearly, there are multiple factors determining the net effect of NO during demyelinating events, and NO may be proved to play distinct roles during different stages of inflammatory demyelination.

The neurotoxicant cuprizone induces demyelination and represents an excellent model system to study components of neuroinflammation. The drug produces an intense and highly reproducible inflammatory reaction within the mouse CNS involving the activation and proliferation of microglia and astrocytes and their...
recruitment to predictable sites during specific stages of treatment. This is a simple system in which T cells are not thought to be involved during the acute inflammatory phase of neuropathology (33–36). This permits a relatively straightforward analysis of glial cell participation in this disease (37–39). To further simplify the model, our laboratory uses a low-dose cuprizone treatment, which results in selective neurotoxicity in the absence of hepatic toxicity (37).

This report applies a genetic approach to decipher the role of iNOS and NO in cellular infiltration and oligodendrocyte death in the cuprizone model of demyelination. We have used iNOSnull (iNOS−/−) mice to show that the induced release of NO does not accelerate disease progression and may serve to temper the severity of pathology.

Materials and Methods

Animals

Breeding pairs of iNOS−/− mice (40) backcrossed onto the C57BL/6 strain and control C57BL/6j mice were housed in a pathogen-free facility at the University of North Carolina (Chapel Hill, NC) and bred on site. All animals were purchased from Jackson Laboratories (Bar Harbor, ME) and handled according to Institutional Animal Care and Use Committee (IACUC) guidelines. All reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. LFB/PAS-stained slides were graded by three independent observers in a double-blind fashion, and the level of pathology was scored on a three-point scale. Higher scores represent greater pathology.

Cuprizone treatment

Animal chow was ground to powder, and cuprizone (bis-cyclohexanone oxalidihydrazone; Sigma-Aldrich, St. Louis, MO) was added to a concentration of 0.2% (w/w) as previously described (37). Animals were provided cuprizone-containing chow ad libidum in a bowl inside their cages and were fed for 3–4 wk (food levels were checked daily). Animals showed no severe side effects of this treatment, although an altered gait, ruffled fur, and lethargic movements were common observations.

Staining

All reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. A modified Luxol fast blue/periodic acid Schiff’s base (LFB/PAS) staining was conducted. Briefly, 4% paraformaldehyde-fixed, parafin-embedded, 5-μM coronal brain sections were rehydrated and immersed overnight at 60°C in 0.2% Solvent Blue 38. Slides were subsequently processed to remove excess stain and to differentiate myelin-specific color. During the initial phases of this study, this differentiation procedure was modified (omission of 70% ethanol) which produced a dramatic enhancement of myelin staining. Sections were then incubated in periodic acid for 5 min, rinsed in distilled water, immersed in Schiff’s base for 15 min, and rinsed under running tap water. Tissue was then stained with hematoxylin (Gills no. 3) for 5 min, washed, dehydrated through 100% ethanol, cleared in Hemo-D (Fisher Scientific, Pittsburgh, PA), and mounted with Permount (Fisher Scientific). LFB/PAS-stained slides were graded by three independent observers in a double-blind fashion, and the level of pathology was rated on a three-point scale. Higher scores represent greater pathology.

Immunohistochemistry

The following Abs were used: rabbit anti-nitrated keyhole limpet hemocyanin polyclonal Ab (anti-nitrotyrosine (N-Tyr), 1/500; Upstate Biotechnology, Lake Placid, NY), rabbit anti-mouse pi isoform of GST (GST-pi; 1/1000; Biotrin, Newton, MA), and rabbit anti-mouse iNOS (1/50; Upstate Biotechnology).

Immunohistochemistry was performed on 5-μM paraffin-embedded sections. Unless otherwise indicated, HBSS was used as the buffer. After rehydration, tissue was processed to enhance Ag availability by “unmasking” the epitopes. Sections were placed in 10 mM citrate buffer (pH 6) and boiled for 5 min in a microwave oven, in plastic Coplin jars. Sections were subsequently incubated (30 min, 37°C) in a buffer containing 5% serum of the host animal of the secondary Ab. For staining with Ricinus communis agglutinin I (RCA-I) lectin (Vector Laboratories, Burlingame, CA), sections were digested with protease K (20 μg/ml, 10 min) in place of microwave-based epitope unmasking. Primary reagents (Abs, lectin) were incubated either at 37°C for 2 h or overnight at 4°C. Sections were then washed and incubated for 30 min at room temperature with the appropriate secondary Ab conjugated with either Texas red, FITC, or 7-amino-4-methylcoumarin-3-acetic acid. For RCA processing, signal was visualized with avidin-Texas red (Vector Laboratories). Slides were mounted with Vectashield medium (Vector Laboratories) and visualized through a microscope (BX-40; Olympus, Melville, NY) (see Imaging).

Immunopositive cells were quantified by counting positive cells within the median of the corpus callosum, confined to a 0.033-mm² area. Only those stained cells with an observable nucleus with 4‘,6’-diamidino-2-phenylindole staining or light microscopy were counted. Cell counts are presented as averages from at least five mice per time point.

Histochemistry for correlates of apoptosis

Sections were processed for the TUNEL assay using a protocol adapted from the manufacturer (Promega, Madison, WI). Briefly, paraffin sections were prepared as for lectin staining, preincubated with equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl (pH 6.6), 200 μM DTT, 250 μg/ml BSA, 2.5 mM CoCl₂) for 10 min at room temperature, and then incubated in TdT reaction mix (180 mM potassium cacodylate, 22.5 mM Tris-HCl (pH 6.6), 100 μM EDTA, 180 μM DTT, 225 μg/ml BSA, 2.3 mM CoCl₂, and 0.2 U/μl terminal deoxynucleotransferase enzyme (Promega or Life Technologies, Rockville, MD)). The fluorophore-conjugated nucleotide was either fluorescein-12-dUTP (5 μM; Roche Diagnostics, Mannheim, Germany), incubated in the presence of 10 μM dATP, or biotin-14-deoxyxycytidine 5’-triphosphate (10 μM; Life Technologies), in the presence of 10 μM deoxyxycytidine 5’-triphosphate. Sections were incubated in a humidified chamber for 1 h at 37°C, rinsed in 2× SSC, and, when applying the biotin-conjugated substrate, exposed to an avidin-fluorophore indicator. TUNEL was the initial procedure when performed in combination with immunocytochemistry; in this case, 2× SSC was used throughout the latter treatment.

Imaging

Stained and processed sections were viewed through an Olympus BX-40 microscope. Images were captured on a video camera (three charge-coupled device video camera system; Optronics Engineering, Goleta, CA) and processed through a red/green/blue framegrabber (CG-7; Scion, Frederick, MD) on a Macintosh 8600 computer (Apple Computer, Cupertino, CA). Video frames were captured through the Scion Image 1.62 or the CG-7 plug-in modules for Adobe Photoshop 4.01 (Adobe Systems, San Jose, CA). Autofluorescing microglia/macrophage were encountered in many paraffin sections from cuprizone-treated animals. These cells were often closely associated with blood vessels, and the signal was manifested as bright blue-white profiles, the product of a broad emission spectrum detected by the triple-band pass filter. Cells or fiber tracts selectively labeled were easily visualized with this background.

Statistical analysis

Data are expressed as mean ± SE. Multiple comparisons were statistically evaluated using a two-tailed Student’s t test. Differences were considered statistically significant if p < 0.05.

Results

iNOS and N-Tyr are elevated in the corpus callosum of cuprizone-treated mice

The activation of iNOS is well documented in MS and in the EAE model; however, its activation in other demyelinating diseases or disease models is less well studied. The corpus callosum was chosen as the site for evaluation because this is a large, well-defined, and heavily myelinated tract, previously shown to produce a consistent and dramatic demyelination in response to cuprizone (37, 38). Wild-type C57BL/6j mice were exposed to cuprizone for 3–4 wk (0.2%, mixed in chow) to evaluate the parameters of the inflammatory reaction associated with demyelination. To determine whether iNOS is activated during cuprizone-induced demyelination, immunohistochemistry for iNOS was performed. iNOS was undetected in the brains of untreated mice but is up-regulated over the course of cuprizone treatment (Fig. 1A). Expression of iNOS colocalizes primarily with microglia/macrophages, but an occasional astrocyte was also found to double label (Fig. 1A).

One of the hallmarks of iNOS activation is the rapid interaction of NO with superoxide radical to form the highly toxic compound peroxynitrite. The nitration of tyrosyl residues is a marker for the presence of peroxynitrite (41), and anti-N-Tyr Abs can be used to...
detect this epitope. Wild-type mice showed intense staining with the N-Tyr Ab at 3.5 wk of treatment, with the predominant N-Tyr-associated immunofluorescence detected in the corpus callosum (Fig. 1B). To determine whether the N-Tyr elevation could be attributed to the presence of NO synthase or neuronal NO synthase, corresponding sections from iNOS/H11002 mice bred to a C57BL/6 background were also studied. Fig. 1C shows that anti-N-Tyr immunoreactivity was all but absent in iNOS−/− mice after 3.5 wk of treatment, indicating that most of the N-Tyr staining could be attributed to iNOS activity. Significant N-Tyr staining was never observed in iNOS−/− mice, even at time points that extended past 3.5 wk of treatment (data not shown).

Cuprizone treatment in iNOS−/− mice reveals a moderately protective role for NO in demyelination

To assess the role of iNOS-derived products in cuprizone-induced demyelination, iNOS−/− mice and wild-type mice were fed cuprizone for 3–4 wk. Brain sections were then processed to display myelin using immunohistochemistry for myelin basic protein (MBP) and LFB/PAS histology (see Materials and Methods). Fig. 2A displays MBP staining over a time course of cuprizone treatment. Fig. 2B shows representative LFB/PAS sections from the forebrain of mice treated for 3.5 wk and a quantitative assessment of this staining conducted in a double-blind manner. Wild-type mice fed 0.2% cuprizone began to show diminished staining for myelin in the midline corpus callosum by 3–4 wk of treatment. In contrast, iNOS−/− mice displayed greater myelin pathology during this timeframe of cuprizone exposure. Subtle differences in myelination between the two groups began to appear 3 wk after treatment; these distinctions were more apparent and consistent by 3.5 wk, but disappeared by 4 wk of treatment. These data indicate that NO does not exacerbate demyelination and may even have an ameliorating role.

Mice lacking iNOS show a greater depletion of oligodendrocytes during cuprizone treatment

Cuprizone-induced inflammation and demyelination are accompanied by a decrease in the numbers of mature oligodendrocytes in the corpus callosum. Staining for GST-pi, a marker shown to be selective for mature myelinating oligodendrocytes (42), shows that iNOS−/− mice undergo a more rapid depletion of oligodendrocytes than wild-type mice (Fig. 3, A and B). By 3.5 wk of treatment there were 50% fewer oligodendrocytes remaining in the corpus callosum in mice lacking iNOS.

To assess whether this decrease in GST-pi+ cells is associated with apoptosis of these cells, rather than dedifferentiation or loss of
markers, tissue sections from wild-type mice were stained for correlates of apoptosis (TUNEL) together with multiple cell-phenotype markers. Fig. 3C, i–iii, displays a section from a 3.5-wk, cuprizone-treated, wild-type mouse processed for apoptosis (TUNEL, Fig. 3Ci), for a marker for mature oligodendrocytes (GST-pi, Fig. 3Cii), and for microglia/macrophage (RCA-I, Fig. 3Ciii). The TUNEL+ cells clearly colocalize with a subpopulation of GST-pi+ cells, and there was no significant cross-reactivity between the TUNEL and RCA stains. A similar lack of cross-staining between TUNEL and glial fibrillary acidic protein (GFAP) was also observed (data not shown). These are compelling observations because a majority of the TUNEL+ cells were found among a vast population of RCA-I+ and GFAP+ cells during the period of maximum cellularity in the midline corpus callosum. These data demonstrate that oligodendrocytes, but not microglia or astrocytes, are undergoing apoptotic death during treatment with cuprizone. Taken with the GST-pi data in Fig. 3, A and B, this suggests that the rapid depletion of oligodendrocytes in the iNOS−/− mice is due to a higher rate of apoptosis occurring in the absence of NO.

Mice lacking iNOS show a similar inflammatory cellular infiltrate during cuprizone treatment

Because the inflammation that occurs during cuprizone treatment is dependent on microglia/macrophage, a potential mechanism through which the lack of iNOS could alter the demyelination is by increasing the number of microglia/macrophages at the site of demyelination. A marker for microglia/macrophage, RCA-1, was used to detect microglia/macrophages in the brains of wild-type and iNOS−/− mice treated with cuprizone over a time course. As shown in Fig. 4A, cells staining positive for RCA-1 rapidly accumulate in the corpus callosum in both wild-type and iNOS−/− mice. A quantitative evaluation of positive cells revealed no differences in numbers of microglia/macrophages in mice lacking iNOS relative to wild-type controls. In addition to staining for RCA-1, an increase in cells staining positive for PAS is observed concomitant with demyelination. This part of the LFB/PAS stain is manifested as a deep magenta deposit and is indicative of actively phagocytic cells. As shown in Fig. 4B, the cellularity in the corpus callosum of iNOS−/− mice increased progressively during treatment at a rate similar to that of wild type. These data indicate that the recruitment of microglia/macrophages was not altered in iNOS−/− mice.

Discussion

This report provides evidence demonstrating that iNOS-derived NO does not exacerbate demyelination and may even have a transient and modest ameliorating effect during an acute neuroinflammatory reaction. We have evaluated a number of inflammatory events—including demyelination, oligodendrocyte depletion and the presence of microglia/macrophage-like cellularity—occurring after cuprizone exposure and have found a consistent increase in the former two measures in mice lacking the iNOS gene. These findings suggest that clinical approaches targeting the reduction of NO levels by the suppression of iNOS or other means should be approached carefully when applied toward the treatment of demyelinating diseases.

Significant levels of iNOS and its products are found in tissue from MS patients (13) and in the animal model for this disease.
EAE (21). Previous studies examining the role of NO in CNS pathology in general, and demyelinating diseases in particular, have produced conflicting results. The use of iNOS inhibitors in EAE resulted in a range of outcomes, from significant protection to exacerbation (23, 25, 27, 28, 32, 43, 44). Studies using either iNOS knockout or knockdown mice have produced mixed results as well (24, 29, 31). The variability of these results has been attributed to the timing of the pharmacological intervention, the nature of the encephalogenic Ag, the route of immunization, the ability of NO to promote or inhibit apoptosis, and the genetic background of the mice. Furthermore, EAE is a complex model involving multiple populations of immune cells, many able to express iNOS or respond to NO. This complexity is reduced in the cuprizone-mediated demyelination model.

This study explored the role of iNOS in the cuprizone model of toxic demyelination, a model with an acute and highly reproducible CNS inflammatory reaction without evidence for T cell involvement. Because T cells are absent, this model permits a more controlled analysis of the contributions of microglia/macrophage-derived NO to inflammation and demyelination. We have found elevated NO release in the corpus callosum (as revealed by the formation of N-Tyr residues) during a neuroinflammatory response in wild-type C57BL/6 mice. The finding of significantly higher levels of N-Tyr staining in wild-type vs iNOS−/− mice indicates that iNOS is the primary source of NO at the site of demyelination. Multiple inflammatory cytokines, including TNF-α and IL-1β are up-regulated after cuprizone treatment and may contribute to the induction of iNOS (45, 46). Our observation that pathology was accelerated in the iNOS−/− mice, animals whose genetic background closely matched the wild-type controls, suggests that iNOS-derived NO plays a protective role in the early phases of the neuroinflammatory response. This difference in demyelination was consistently observed at early timepoints and disappeared by 4 wk of treatment, indicating that the presence of NO is important in the early stages of pathology but is not sufficient to fully protect against demyelination. The increased rate of oligodendrocyte cell loss during the earlier stages of cuprizone treatment in the iNOS−/− mice indicates that factors other than (or in addition to) NO could be mediating this toxicity. This finding contrasts with previous in vitro studies indicating that oligodendrocytes are the most sensitive of all glial populations to the toxic effects of NO (47). The extended viability of oligodendrocytes in the presence of...
NO is also reflected in the fact that myelin in corpus callosum remained intact for a longer period in the wild-type mice, suggesting that iNOS-derived NO can play a protective role during certain stages of a demyelinating process.

One potential explanation for our findings is that the ultimate impact of NO during inflammation is via its indirect influence on processes that could contribute to the viability of oligodendrocytes. Perhaps the most compelling role for NO in this vein would be as a global mediator of the inflammatory cascade; however, we observed that the inflammatory infiltrate during cuprizone treatment was independent of the presence of iNOS. This suggests that NO is not involved in the accumulation of microglia/macrophages at the site of demyelination, though NO has previously been shown to down-regulate macrophage proliferation (48). However, the role of NO in inflammation may have less to do with an effect on numbers of microglia than with their state of activation, as it has been previously shown that NO can down-regulate inflammatory cytokine production (49). Interestingly, recent papers have shown a protective role of iNOS in an autoimmune disease model, EAE, where the lack of iNOS results in an earlier onset and more aggressive disease (29, 31, 50, 51). Although the role of iNOS and NO in EAE remains controversial, our data support the view that NO may serve a protective function in pathologies involving a clear inflammatory component.

In summary, this study shows that the elimination of iNOS does not ameliorate and may exacerbate demyelination during exposure to the neurotoxicant cuprizone. This is the first documentation of such a role for iNOS in a nonautoimmune demyelinating model and may have ramifications for the treatment of the diverse group of demyelinating diseases. Our data suggest caution in the consideration of inhibitors of iNOS or NO for the clinical treatment of demyelinating disease.

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