Role of Nitric Oxide as Mediator of Nerve Injury in Inflammatory Neuropathies

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Abstract
Different lines of evidence suggest that nitric oxide (NO) plays a key role in the pathogenesis of inflammatory neuropathies; however, it is still unclear which structures in the peripheral nerve are the primary targets of NO-mediated nerve injury. To address this issue, we determined the expression of NO metabolites in sural nerve biopsies and in cerebrospinal fluid from patients with inflammatory neuropathies and studied the pathologic effects of NO in an in vitro model of myelinated Schwann cell-neuron cocultures. In cerebrospinal fluid samples, nitrite levels remained unaltered; however, nitrotyrosine, a marker for peroxynitrite formation, could be identified in nerve biopsies from patients with inflammatory neuropathies. In an in vitro model of Schwann cell neuron cocultures, high concentrations of NO induced robust demyelination, which was the result of NO-mediated axonal injury, whereas Schwann cell viability remained unaffected. These findings suggest that in contrast to Schwann cells, sensory neurons are the primary target of NO-mediated cytotoxicity and the loss of myelin is the result of selective damage to axons rather than a direct harmful effect to Schwann cells. Our findings imply that NO contributes to the pathologic changes seen in the inflamed peripheral nervous system, which is characterized by the features of axonal injury and subsequent myelin degradation, previously described as Wallerian-like degeneration.

Key Words: Axon, Demyelination, Guillain-Barré syndrome, Inflammation, Inflammatory neuropathy, Nitric oxide, Peripheral nerve, Schwann cell.

INTRODUCTION
Inflammatory neuropathies comprise a heterogeneous spectrum of clinical entities including the Guillain-Barré syndrome (GBS) with its demyelinating (acute inflammatory demyelinating neuropathy) and axonal variants (acute motor axonal neuropathy, acute motor and sensory axonal neuropathy) as well as different types of chronic inflammatory demyelinating neuropathy (CIDP) (1–4). Although the variability of the clinical features and courses reflects substantial differences in the pathogenesis, it is commonly accepted that proinflammatory components are crucial effectors in mediating cellular damage in all subtypes of this group of neuropathies. Of those, the short-lived free radical nitric oxide (NO) has been invoked as an important effector of nerve injury (5–9). In an inflammatory setting, NO can react with superoxide (O_2^-) to form peroxynitrite (ONOO^-) (10), an oxidant that has been implicated in promotion of cytotoxicity (11). NO is synthesized from the amino acid l-arginine by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS have been characterized: endothelial, neuronal, and inducible NOS (iNOS), expressed mainly in leukocytes (9). In experimental allergic neuritis, an animal model of acute immune-mediated peripheral neuritis, iNOS can be detected in endoneural macrophages and other inflammatory cells that are localized in the nerve roots where prominent demyelination is found (12). Besides inflammatory cells, which probably represent the main source of NO, Schwann cells, when activated by inflammatory stimuli, can release NO as well (12, 13).

Although these findings strongly support a pathogenic role of NO during nerve injury, it still remains unclear which compound of the peripheral nerve, in particular, which cell type, is the primary target of NO-mediated toxicity. There is evidence from autopsy studies in GBS that both myelinating Schwann cells as well as ensheathed axons are exposed to NO-releasing macrophages at sites of inflammation (14–16). However, studies that examined harmful effects of NO to either Schwann cells or axons are few and yielded controversial results (7, 9, 13, 17, 18). We sought to clarify this question by determining the expression of NO metabolites in sural nerve biopsies and in cerebrospinal fluid (CSF) of patients with inflammatory neuropathies and by examining the effects of NO in a cell culture model consisting of dorsal root ganglia (DRG) neurons and myelinating Schwann cells.

MATERIALS AND METHODS
Immunohistochemistry for 3-Nitrotyrosine
Sural nerve biopsies (n = 8) were obtained for diagnostic purposes with informed consent from patients (19).

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For the detection of reactive nitrogen species within the inflamed peripheral nervous system (PNS), biopsies from patients classified as having CIDP (n = 4) and based on accepted research criteria were studied. None of the patients had received any immunomodulatory or immunosuppressive treatment within 3 months before biopsy. Samples from patients diagnosed as having hereditary neuropathies (n = 4) represented noninflammatory controls. Immunohistochemistry for 3-nitrotyrosine was performed on deparaffinized sections, as described previously (11), using a rabbit polyclonal anti-nitrotyrosine IgG antibody (1:500 in blocking solution; Upstate Biotechnology, Lake Placid, NY) or antigen-competed primary antibody or blocking solution without primary antibody. A biotinylated anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) made in goat was used as a secondary antibody. For double-labeling studies, sections were incubated with rabbit anti-nitrotyrosine IgG antibody and antibody against neurofilament (SMI31; Sternberger Monoclonals, Lutherville, MD) or against myelin basic protein (MBP) (SMI94, 1:500, 2 hours; Sternberger Monoclonals) followed by specific secondary antibodies conjugated to different fluorophores (1:200, 1 hour, room temperature).

**NO₂⁻ Activity in Human Cerebrospinal Fluid and Cell Supernatant**

Human CSF samples were obtained with informed consent from patients with GBS (n = 18) and CIDP (n = 6) diagnosed according to established criteria (2, 3, 20). The average cell count was 3 cells/μL (range 1–18), and the CSF total protein was 1.08 g/L (range 0.36–3.00). CSF samples from patients with inflammatory (n = 13) and noninflammatory neurologic diseases (n = 7) with an average cell count of 5 cells/μL (range 1–32) and a total protein of 0.47 g/L (range 0.29–0.94) served as controls. After lumbar puncture, CSF samples were centrifuged immediately at 3,000 × g for 10 minutes; supernatants were aliquoted and stored at −70°C. NO release in CSF samples and cell supernatant was assessed by measuring nitrite (NO₂⁻), the stable oxidation product of NO, by a modified Greiss assay. Briefly, 75 μL of samples (CSF 1:10 diluted in PBS) and standards (NaNO₂) were applied to a standard 96-well plate. Equal volumes of 2% sulfanilamide in 5% H₃PO₄ and 0.2% naphthylethylene-diamine were mixed, and 25 μL of this mixture was added to each well. After 10 minutes at room temperature, optical densities were measured at 540 nm on an ELISA plate reader (Multiscan; Thermo Electron, Waltham, MA). To determine NO scavenger activity of the CSF samples, 25 μL of 10 μmol/L DETANONOate was added to each sample, and NO activity was measured as described.

**Myelinating Cultures**

Myelinating cultures were prepared as described previously (21). DRGs were harvested from embryonal day 15 (E15) rat pups. After trypsinization, DRGs and accompanying endogenous Schwann cells were plated on collagen-coated plastic dishes, treated for 24 hours with cytosine arabinoside (10 μmol/L), and maintained for 5 days in neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 1% fetal bovine serum (FBS) (HyClone, Logan, UT), 2 mol/L i-glutamine, 2% B27 serum-free supplement (Invitrogen) and 100 ng/mL nerve growth factor (NGF) (Sigma-Aldrich, St. Louis, MO). No further exogenous Schwann cells were added to the cultures. Myelination was initiated by switching from neurobasal medium to Eagle’s medium with Earle’s salts containing 15% FBS, 10 ng/mL NGF, and 50 μg/mL ascorbic acid (Sigma-Aldrich). Cultures were maintained in myelinating medium for 21 days before experiments were started.

**Myelin Quantification**

Myelin segments were visualized by Sudan black staining as described previously (22). Whole culture dishes were fixed with 4% paraformaldehyde and postfixed with 0.1% OsO₄ for 45 minutes. Cultures were then dehydrated and stained with 0.5% Sudan black in 70% ethanol for 1 hour, washed with ethanol, and mounted in glycerin jelly. For myelin quantification, nine randomly selected pictures in low magnification (20×) were obtained, and the lengths of all internodal segments were measured as described (23). Data are expressed as averages of the total length of myelinated internodes per field and the average of myelinated internodal segments. In a subset of experiments, the cell cultures were double stained with a monoclonal antibody against MBP (SMI94) followed by an rabbit anti-neurofilament antibody (Sigma-Aldrich) and the extent of myelination was quantified by measuring the whole length of MBP-positive segments in each of nine randomly selected fields per well. Both methods were correlated, and results corresponded well with each other.

**Preparation of Dissociated Neuronal Cell Cultures**

Neuronal cell cultures were prepared from E15 rat DRG neurons as described previously (24). Whole ganglia were trypsinized, dissociated, and plated on collagen-coated glass coverslips (Fisher Scientific, Pittsburgh, PA) at low concentrations in 24-well plates (Becton Dickinson, Franklin Lakes, NJ). Neuronal cultures were maintained in neurobasal medium containing 1% FBS, 2 mol/L i-glutamine, 2% B27 serum-free supplement, and 100 ng/mL NGF.

**Neuronal Cell Survival and Neurite Outgrowth Assay**

The cells were plated, and after 30 minutes the different agents were added. The NO donor DETANONOate was obtained from Alexis Biochemicals (Nottingham, UK) and the NO scavenger agent 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO) from Calbiochem (San Diego, CA). After 20 hours of incubation, the cells were fixed with 4% paraformaldehyde and stained with a neuron-specific antibody against III-β tubulin (1:5,000; Promega, Madison, WI) followed by an Alexa Fluor conjugated secondary antibody (1:200; Molecular Probes, Eugene, OR). Neuronal survival and neurite outgrowth was quantified using IMAGE J, a public domain image-processing program (http://rsb.info.nih.gov/ij/). Images were acquired in

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low magnification (20×) from randomly selected fields (n = 8–12) and the longest neurite of 60 to 100 neurons per each condition was measured, as described elsewhere (25, 26). Cell clusters of extreme density and neurons without neurites were rejected. To determine neuronal survival, all III-β tubulin-positive cells were counted as described elsewhere (8). Each experimental condition was tested in duplicate wells and repeated two to three times with neurons from a different set of animals. Neurite length is expressed as a percentage of the mean neurite length ± standard error in the control group for at least two independent experiments.

Preparation of Schwann Cells and Cytotoxicity Assay

Schwann cells were prepared by a modified Brockes’ method (27). Briefly, anesthetized neonatal rats were killed by decapitation, and sciatic nerves were dissected. After digestion with 0.1% collagenase (Worthington, Lakewood, NJ) and 0.25% trypsin, cells were plated in Dulbecco’s modified Eagle’s medium with 10% FBS. To eliminate fibroblasts, the cells were treated with two cycles of cytosine arabinoside (10 μmol/L) followed by complement lysis with anti-thymidine 1.1 antibody. The resulting cultures, Schwann cells at >95% purity, were expanded by medium with forskolin. Schwann cell viability was determined by a Live-Death Viability/Cytotoxicity Kit (Molecular Probes) according to the manufacturer’s instructions. After treatment with 1 mmol/L DETANONOate, cell viability was determined by dividing the number of all living cells by the number of all propidium iodide-positive dead cells in 12 randomly selected fields from two separate wells for each condition. Schwann cells that were lysed with 0.1% Triton X-100 served as positive controls.

RESULTS

Immunohistochemical Studies of Sural Nerve Sections

All tissue sections from patients with CIDP showed active disease with foci of inflammatory cells, whereas in the hereditary neuropathy group inflammatory infiltrates were not observed (data not shown). Nitrotyrosine residues on proteins were detected within the endoneurium. In nerve sections from patients with CIDP, a patchy staining pattern of axons and accompanying Schwann cells was observed (Fig. 1A, B). Further, 3-nitrotyrosine-positive staining was

FIGURE 1. Immunohistochemical staining of nitrotyrosine in the inflamed peripheral nerve. (A) Nitrotyrosine-positive cells can be depicted in the endoneurium of patients with chronic inflammatory demyelinating polyneuropathy (CIDP), with positive immunoreactivity morphologically localized to axons (arrows), Schwann cells (arrowhead), and monocytes (rhomb). (B) Sections from patients with noninflammatory neuropathies did not reveal any positive immunoreactivity (original magnification: 400×). (C–H) Nerve sections from patients with inflammatory neuropathies are double labeled with antibody against nitrotyrosine (green, C, F), myelin basic protein (red, D) or neurofilament (red, G). Colocalization of the two labels confirms nitrotyrosine exposure of Schwann cells (E) and axons (H) (original magnification: 400×).
also seen in inflammatory cells within the nerve fibers. Control immunohistochemical analyses showed background staining only. By double labeling, 3-nitrotyrosine-positive staining was observed in MBP-positive myelinating Schwann cells (Fig. 1C–E) as well as in sensory axons (Fig. 1F–H). There was no obvious difference in the staining distribution or intensity between Schwann cells and axons.

Patients with Inflammatory Neuropathies Do Not Show Increased Intrathecal Levels of NO$_2^-$

The production of NO$_2^-$, which partially reflects NO release, was assessed in CSF samples from 18 patients with GBS and 6 patients with CIDP. All samples showed comparable low levels of NO$_2^-$ with no difference between patients with an inflammatory neuropathy and control subjects (Fig. 2). To investigate the possibility that the enhanced NO$_2^-$-mediated cytotoxic effects in the inflamed PNS could represent the result of a reduced capability to scavenge injuriously high levels of NO$_2^-$, the production of NO$_2^-$ by the NO donor DETANONOate was measured in the presence of CSF from patients with GBS and CIDP. Although the addition of DETANONOate resulted in a 10-fold increase of NO$_2^-$, neither the CSF from patients

**FIGURE 2.** Cerebrospinal fluid (CSF) samples from control subjects with inflammatory neurologic diseases (IC), control subjects with noninflammatory neurologic diseases (NIC), Guillain-Barré syndrome (GBS), and chronic inflammatory demyelinating neuropathy (CIDP) have similar levels of NO$_2^-$. CSF from patients with inflammatory neuropathies or from control subjects does not attenuate the release of NO$_2^-$ by DETANONOate.

**FIGURE 3.** Sudan black-stained myelinated Schwann cell-neuron cultures in the presence of 1 mmol/L DETANONOate (A) showed a decrease of darkly stained myelin segments (arrowheads) in contrast to cultures treated with vehicle only (B). Scale bars = 100 μm. (C) Exposure of myelinated Schwann cell-neuron cocultures to higher doses of the NO donor DETANONOate led to a significant decrease in total myelin per well (*, p < 0.05, Mann-Whitney U test). (D) Double immunostaining of myelinated Schwann cell-neuron cocultures against neurofilament (green) and myelin basic protein (red). Scale bar = 100 μm. (E) In myelinated Schwann cell-neuron cultures treated with DETANONOate, a prominent reduction of MBP-positive internodal segment length can be found, indicating loss of myelination (**, p < 0.001, Mann-Whitney U test). (F) Myelinated Schwann cell-neuron cocultures, which were exposed to NO, showed significant shorter internodal segments (*, p < 0.05; **, p < 0.001; Mann-Whitney U test).
with inflammatory neuropathies nor the CSF from control subjects with inflammatory or noninflammatory diseases attenuated NO₂⁻ production (Fig. 2).

**High Concentrations of Nitric Oxide Cause Significant Loss of Myelin in Vitro**

Because our immunohistochemical studies indicated that nerve fibers are exposed to increased levels of NO during inflammation, we examined the effect of NO on myelinated Schwann cell-neuron cocultures. Dissociated DRG neurons and Schwann cells were cultured and maintained for 28 days in medium containing ascorbic acid. In this cell culture model, Schwann cells and neurons form an intimate association that allows Schwann cells to interact with sensory axons and to initiate the formation of myelin. After 28 days, Schwann cells have ensheathed the axons and the compact myelin sheaths can be visualized by immunocytochemistry (Fig. 3D). The slow NO donor DETANONOate was added to myelinated cultures; 24 hours later, cultures were fixed, and the amount of myelin was quantified by Sudan black staining. The cultures showed a dose-dependent loss of the total amount of myelin at higher concentrations of DETANONOate (Fig. 3A–C). Further, DETANONOate-treated cell cultures also had a significantly reduced average myelinated internodal segment length (Fig. 3F). In an independent subset of experiments the degree of myelination was also assessed by quantifying the MBP-positive segments, which showed results comparable to those with the Sudan black-based assay (Fig. 3D, E).

**DRG Neurons and Schwann Cells Show Different Susceptibility to NO-Mediated Cytotoxicity**

Because there are two major compounds in the myelinated nerve fibers, Schwann cells and ensheathed axons, the decrease in myelin might reflect potential effects of NO on either cell type. Therefore, we tried to evaluate which cells represent the primary target of NO-mediated tissue damage. To determine the effects of NO on Schwann cells, the viability of pure Schwann cell cultures after exposure to DETANONOate was assessed by a fluorescence-labeled live-dead assay and by counting the total number of cells. Exposure to the NO donor in the same concentrations that caused significant demyelination in Schwann cell-neuron cocultures did not affect cell viability or morphology of pure Schwann cell cultures (Fig. 4A).

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**FIGURE 4.** (A) Schwann cells treated with high doses of DETANONOate (1 mmol/L) did not show enhanced cell death compared with cell cultures treated with vehicle alone. (B) NO leads to axonal injury. Dissociated sensory neurons exposed to DETANONOate showed a dose-dependent axonal outgrowth arrest (***, p < 0.001, Mann-Whitney U test). (C) Conditioned medium (CM) from myelinated Schwann cell-neuron cocultures did not prevent NO-mediated neurite outgrowth inhibition, whereas the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) attenuated the inhibitory effect of NO on axonal outgrowth (**, p < 0.001 from the DETANONOate 0.5 mmol/L group, Mann-Whitney U test).
To study the selective effect of NO on axons we used dissociated DRG that were cultured with a high concentration of NGF (100 ng/mL), a dose that robustly protects against NO-mediated cell death (28). Neuronal survival in the presence of NO was assessed by counting all III-β tubulin-positive cells. As expected, exposure of sensory neurons to DETANONOate in the presence of NGF did not lead to significant cell death (data not shown). However, in contrast to their effects on neuronal viability, NO had a dramatic effect on axonal outgrowth in dissociated DRG neurons. At this age, sensory neurons have a strong capability to extend neurites, especially during the first 24 hours in culture. In the control cultures, most neurons displayed long neurites that were at least 60 μm in length. In contrast, by adding DETANONOate to the cultures, we observed a prominent inhibition of neurite outgrowth (Fig. 4B). This neurite outgrowth inhibition was dose dependent. Even low concentrations of DETANONOate, which did not cause significant demyelination or neuronal cell death in the previous experiments, resulted in a significant neurite outgrowth arrest. At higher doses almost no neurite extensions could be detected. To confirm the specific effect of NO on the inhibition of neurite outgrowth we used the chemical NO scavenger PTIO to prevent NO-mediated axonal injury. As shown in Figure 4C, low doses of PTIO effectively prevented the inhibitory effect of NO on neurite outgrowth. Next, we asked whether putative soluble factors released during the process of myelination could salvage axons from NO-mediated toxicity. Thus, myelinated Schwann cell-neuron cocultures were maintained in serum-free medium for 24 hours, and supernatants were collected. Seventy-five microliters of conditioned medium from myelinated Schwann cell cocultures were added to the wells before they were exposed to NO. Even at higher concentrations, no protective effect of conditioned cell medium from myelinated cell cultures was discerned (Fig. 4C).

**DISCUSSION**

**Role of NO in Demyelination and Axonal Injury**

In this study, we demonstrated that nerve fibers from patients with inflammatory neuropathies are exposed to increased levels of NO metabolites. Further, in a cell culture model of myelinated DRG neurons, the NO donor DETANONOate led to a dose-dependent loss of myelin, as indicated by a decrease of the total amount of myelin and shorter myelinated internodal segments. Although in vivo Schwann cells and sensory neurons are both exposed to increased levels of NO, in vitro NO mediates substantial cell damage only in sensory neurons but not in Schwann cells. These findings imply that the observed loss of myelin is the result of axonal injury rather than NO-mediated toxicity to Schwann cells.

NO has been reported to induce cell death in a variety of glial cells and different neurons (6–8, 17, 29, 30). Of note, most of these studies have investigated the pathologic effects of NO in the context of inflammatory diseases of the central nervous system, whereas studies on the role of NO in immune-mediated neuropathies are less numerous. During inflammation, macrophages and activated glia cells release NO after the induction of iNOS. In GBS, macrophages represent the major cell population in inflammatory infiltrates of the spinal roots (1, 2, 31). Because NO is known to be an important effector molecule of macrophage-mediated tissue damage, its effects on cells of the PNS are of particular interest. This importance is further underlined by studies in experimental allergic neuritis, in which enhanced expression of iNOS can be detected in demyelinated nerve roots but not in the sciatic nerve where axonal degeneration is present (12). Similarly, the administration of NO inhibitors may attenuate some, but not all, variants of experimental allergic neuritis (32). Although these observations point to an important role of NO in the pathogenesis of inflammatory neuropathies, it remains unclear which cells represent the primary targets of NO in myelinated nerve fibers.

In vitro studies indicate that generally both sensory neurons and Schwann cells could be affected by NO-mediated cell injury. Smith and colleagues demonstrated that exposure to NO induces conduction block and axonal degeneration in dorsal and ventral roots in vitro (6, 7). The axonal outgrowth arrest observed in our cell culture model after NO administration confirms these reports and further supports the concept that axons are the primary targets in NO-mediated toxicity in the PNS. It is still a matter of debate how NO-associated cytotoxicity and axonal injury are mediated. In addition to induction of apoptosis (33), excitotoxicity (34) and inhibition of mitochondrial respiration (35) have been postulated. Furthermore, the p38 mitogen-activated protein kinase has been proposed as a NO-activated positive signal for axonal destruction (8). The aim of our study was not to unravel the underlying mechanism of NO-mediated axonal injury. Thus, apart from our identification of the cellular target of NO, we cannot exclude or favor any intracellular target of the postulated pathophysiologic pathways.

The effects of NO on Schwann cell viability are controversial. Although Schwann cells may exert immunoregulatory functions within the inflamed PNS by secretion of nitric oxide metabolites upon activation by proinflammatory cytokines, they may also undergo cell death in the presence of NO (13, 17). Moreover, NO may even be a stimulus for trophic support by Schwann cells. Keswani et al (36) demonstrated that erythropoietin, which prevents neuronal cell death and axonal degeneration, is expressed by Schwann cells in the presence of moderately elevated levels of NO. In contrast with these data and our observations, Nagano et al (17) have proposed that NO can act as a proapoptotic effector in an immortalized Schwann cell line. Differences in the cell culture (immortalized Schwann cell line versus primary cell culture) and in the culture methods may explain such discrepancies.

Surprisingly, we could not overcome the inhibition of axonal outgrowth by adding conditioned medium from myelinated cocultures to neurons, which were exposed to NO. Schwann cells are known to generate a variety of factors important for maintenance and trophic support of myelinated and unmyelinated axons. These include soluble...
growth factors (37) but also axonoprotective membrane-bound glycoproteins such as erythropoietin (36). Although soluble factors may prevent axonal degeneration to some degree, our findings imply that they alone are insufficient to rescue neurons from injury caused by excessive levels of NO. In contrast, our finding that PTIO, a chemical NO radical scavenger, could rescue axons from outgrowth arrest argues that specific agents, which are able to chemically neutralize NO, are necessary to save axons from NO-mediated degeneration.

**NO Metabolites in Nerve Biopsies and in the Cerebrospinal Fluid of Patients With Inflammatory Neuropathies**

The expression of NO metabolites was determined in sural nerve biopsies and in CSF samples from patients with GBS and CIDP. Only in nerve biopsies from inflammatory neuropathy patients did axons, Schwann cells, and monocytes stain positively for 3-nitrotyrosine, indicating the exposure of these cells to reactive, potentially cytotoxic NO metabolites in this pathologic situation. Our double-labeling studies again demonstrate that both myelinated Schwann cells as well as ensheathed axons were exposed to nitrotyrosine, indicating increased oxidative stress in both cell types. The cellular sources of NO within the inflamed PNS have been extensively described before and were therefore not studied further. A large body of evidence points to monocytes/macrophages (1, 12, 38), present in high numbers in the inflamed peripheral nerve, and Schwann cells (13) as the predominant source of NO metabolites. In contrast to our immunohistochemical findings, the levels of the NO metabolite NO$_2^-$ were not elevated in the CSF from patients with inflammatory neuropathies. Previously, increased NO$_2^-$ production in the CSF has been demonstrated in other inflammatory-driven neurologic diseases including multiple sclerosis (39, 40) and bacterial meningitis (41, 42). In multiple sclerosis, increased NO$_2^-$ levels in the CSF were correlated with the volume of gadolinium-enhanced lesions on magnetic resonance imaging and associated with disease progression (40). Because of the absence of a blood-nerve barrier in the dorsal roots, the sites of inflammation in GBS are directly exposed to the CSF space, which raises the possibility that NO$_2^-$, as the stable metabolite of NO, might also be increased in the CSF in patients with GBS and other inflammatory neuropathies. However, in line with a previous report (43), NO$_2^-$ levels in the CSF were unaltered in patients with GBS. Similarly, NO$_2^-$ levels in the CSF from patients with CIDP were not increased either. To our knowledge this is the first study that has examined the intrathecal production of NO metabolites in patients with CIDP. An explanation for the unchanged intrathecal NO$_2^-$ levels in GBS and CIDP might be the relatively small molecular size of this metabolite, which could facilitate unrestricted transmigration through the blood-nerve barrier and equalization of different compartmental NO$_2^-$ levels. Another explanation could be that in inflammatory diseases of the peripheral nervous system the amount and proportion of tissue (i.e. nerve roots) that is in contact with CSF is relatively small compared with the parts of the CNS in multiple sclerosis or meningitis. Although local NO exposure is evident, it may not suffice to alter NO metabolite levels in the large CSF compartment. Furthermore, we conclude that tissue damage in GBS is probably not caused by a reduced capability to scavenge injuriously high levels of NO, as determined by our CSF adding back experiments with DETANONOate as shown in Figure 2.

**Relevance to the Pathogenesis of Inflammatory Neuropathies**

Our present data provide further evidence for an important role of NO in the pathogenesis of inflammatory neuropathies and support current immunopathologic concepts in patients with GBS and CIDP. In the acute and chronic forms of inflammatory demyelinating neuropathies, demyelination is typically located in the spinal roots but also occurs along the peripheral nerves and nerve terminals (31, 44). In addition to demyelination, axonal damage is frequently observed in autopsy and pathologic studies on sural nerve biopsies in both acute and chronic forms of inflammatory neuropathies, especially in patients with severe disease courses and fatal clinical outcomes (45–47). This damage is highlighted in cases of acute motor neuropathy and acute motor and sensory neuropathy, in which the most prominent pathologic hallmark is axonal degeneration without substantial inflammation and demyelination (48). In those cases, immunopathologic studies demonstrated the presence of macrophages within the periaxonal space, which are surrounded by an intact myelin sheath and unaffected Schwann cells (15, 48, 49).

NO, as a key effector molecule of macrophages, might be a good candidate for mediating cell injury, inasmuch as infiltrating macrophages are prominent features in both demyelinating and axonal variants of GBS. Our data support the concept that moderate levels of NO released by macrophages may induce substantial axonal injury without affecting the myelin-forming Schwann cells. The observation that demyelination occurs as a consequence of NO-mediated axonal injury reflects the frequently observed finding of Wallerian-like degeneration in inflammatory neuropathies, which means the sequential features of axonal breakdown and myelin degradation by Schwann cell dedifferentiation. This concept does not exclude other mechanisms of axonal damage and demyelination mediated by macrophages and humoral factors (i.e. complement or ganglioside antibodies). Conversely, it is likely that the active stripping of myelin by macrophages requires a whole cascade of proteases and lysosomal enzymes, which may have divergent effects on axons and Schwann cells than NO.

Because a significant proportion of patients with GBS and CIDP have a poor clinical outcome with incomplete functional recovery, which reflects severe axonal damage, the identification of mechanisms responsible for axonal injury is of particular interest. Our data support the notion that NO is critically involved in the process of axonal degeneration in the inflamed PNS. However, further studies need to identify factors that could salvage axons from NO-mediated axonal degeneration.
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