

Peroxynitrite-Induced Oligodendrocyte Toxicity Is Not Dependent on Poly(ADP-Ribose) Polymerase Activation

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ABSTRACT Oligodendrocyte loss is a characteristic feature of several CNS disorders, including multiple sclerosis (MS) and spinal cord injury. However, the mechanisms responsible for oligodendrocyte destruction remain undefined. As recent studies have implicated peroxynitrite in the pathogenesis of both spinal cord injury and MS, we have examined whether peroxynitrite may mediate at least some of the oligodendrocyte damage and demyelination observed in these conditions. Primary rat oligodendrocytes were exposed to authentic peroxynitrite *in vitro* and assessed for cytotoxicity. Mitochondrial function, measured by the reduction of MTT to formazan, and mitochondrial membrane potential were used as indicators of cell viability. Cell death was quantitated by measuring either the release of lactate dehydrogenase from, or the uptake of propidium iodide into, damaged and dying cells. Peroxynitrite dose-dependently reduced the viability of primary oligodendrocytes and induced cell death. Furthermore, peroxynitrite significantly increased DNA strand breakage and the activity of poly(ADP-ribose) polymerase (PARP) in oligodendrocyte cultures. To identify whether PARP activation plays a role in peroxynitrite-induced oligodendrocyte toxicity, we examined the effects of the PARP inhibitors 3-aminobenzamide (3AB) and 5-iodo-6-amino-1,2-benzopyrone (INH₂BP) on mitochondrial function and cell death in oligodendrocytes. The presence of 3AB and INH₂BP did not protect oligodendrocytes from peroxynitrite-induced cytotoxicity. However, both compounds significantly reduced PARP activity in these cells. Primary oligodendrocytes generated from PARP-deficient mice were also highly susceptible to peroxynitrite-induced cell death. Therefore, our results show that peroxynitrite exerts cytotoxic effects on oligodendrocytes *in vitro* independently of PARP activation. *GLIA* 41:105–116, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION

Multiple sclerosis (MS) is a central nervous system (CNS) disorder characterized by oligodendrocyte destruction and demyelination (Sobel, 1995), with damage occurring to mature oligodendrocytes as well as remyelinating cells (Prineas, 1975). Other conditions affecting the CNS, such as spinal cord injury, are also associated with a loss of both oligodendrocytes and myelin (Blight, 1985; Rosenberg and Wrathall, 1997;

Casha et al., 2001; Grossman et al., 2001). Although common mechanisms may be responsible for the oligodendrocyte damage and demyelination observed in

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these disorders, the precise effector molecules have yet to be established (Merrill and Scolding, 1999). Some investigators have suggested that oligodendrocyte destruction may be a consequence of the release of cytokines during CNS inflammation (Brosnan et al., 1988), whereas other workers have proposed that oligodendrocytes are sensitive to glutamate-induced toxicity (Matute et al., 2001). More recently, attention has focused on the role of free radicals in mediating oligodendrocyte injury (Smith et al., 1999).

Several reports have demonstrated that the free radical nitric oxide (NO) induces oligodendrocyte death *in vitro* (Merrill et al., 1993; Mitrovic et al., 1994a, 1994b, 1995, 1996). Furthermore, *in vivo* evidence exists to support a role for NO in the pathogenesis of spinal cord injury, as well as MS and its animal model, experimental allergic encephalomyelitis (EAE) (Giovannoni et al., 1998; Liu et al., 2000; Xu et al., 2001). Many of the cytotoxic actions previously attributed to NO are now known to be mediated through peroxynitrite, the reactive oxidant formed when NO and superoxide combine (Hausladen and Fridovich, 1994; Zingarelli et al., 1996). Therefore, oligodendrocyte damage in certain CNS conditions may result from peroxynitrite rather than NO. It is noteworthy in this respect that peroxynitrite production has been observed in CNS tissues from MS patients (Bagasra et al., 1995; Hooper et al., 1997; Cross et al., 1998; Liu et al., 2001), EAE-diseased animals (Cross et al., 1997; Van der Veen et al., 1997; Hooper et al., 2000; Scott et al., 2001), and spinal cord-injured rats (Scott et al., 1999; Liu et al., 2000; Xu et al., 2001). Moreover, peroxynitrite was recently shown to damage CNS myelin *in vivo* (Touil et al., 2001).

Peroxyntirite can induce a variety of toxic interactions, including tyrosine nitration (Ischiropoulos et al., 1992b), lipid peroxidation (Radi et al., 1991), and inhibition of mitochondrial respiration (Hausladen and Fridovich, 1994; Bolaños et al., 1995). In addition, peroxynitrite may cause cell death via DNA single-strand breakage and the subsequent activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP; EC 2.4.4.30). Once activated, PARP catalyzes the attachment of ADP-ribose units from its substrate, nicotinamide adenine dinucleotide (NAD⁺), to various proteins (Ueta and Hayashi, 1985). However, excessive activation of PARP depletes intracellular NAD⁺ levels, slowing the rate of glycolysis, electron transport, and ATP formation, which eventually leads to cell death through energy failure mechanisms (Szabó, 2000).

Activation of PARP has previously been shown to play a role in neurotoxicity (Cosi et al., 1994; Zhang et al., 1994). More specifically, several studies have implicated PARP activity in the pathogenesis of various neurodegenerative disorders (Love et al., 1999; Mandir et al., 1999). We have recently demonstrated PARP activation in CNS tissues from spinal cord-injured rats and EAE-diseased animals (Scott et al., 1999, 2001). Therefore, we postulate that in spinal cord injury and MS, oligodendrocyte death may occur as a result of

peroxynitrite production and activation of PARP. In the present study, we have examined whether native peroxynitrite exerts cytotoxic effects on primary oligodendrocytes *in vitro*. Once we had established that peroxynitrite induces oligodendrocyte death, we then went on to assess the nature of peroxynitrite-mediated toxicity, with particular emphasis on the role of PARP activation in this process.

MATERIALS AND METHODS

Cell culture reagents were obtained from GIBCO Life Technologies (Grand Island, NY). Antigalactocerebroside antibody and antiglial fibrillary acidic protein were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxynitrite and 5-iodo-6-amino-1,2-benzopyrone were obtained from Alexis Biochemicals (San Diego, CA). 3,3'-dihexyl-oxocarbocyanine iodide [DiOC6(3)] and propidium iodide were purchased from Molecular Probes (Eugene, OR). [³H]NAD⁺ was obtained from Dupont-NEN (Boston, MA). ScintiSafe Plus scintillation liquid was purchased from Fisher Scientific (Pittsburgh, PA). The cell death detection ELISA^{PLUS} assay kit was obtained from Roche Diagnostics (Indianapolis, IN). All other reagents were obtained from Sigma Chemical.

Preparation of Primary Rat Oligodendrocyte Cultures

Oligodendrocytes were prepared from the cerebral cortices of 2- to 8-day-old Wistar rats as previously described with minor modifications (Mitrovic et al., 1994a). In brief, the brains and meninges were removed and the cortices minced and dissociated in 0.25% trypsin. The resulting cell suspension was washed once and cells were plated into 175 cm² flasks. Cells were cultured in medium consisting of Dulbecco's modified Eagles medium supplemented with 15 mM HEPES, 1 mM sodium pyruvate, 10% fetal calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. Oligodendrocytes were harvested by brief mechanical dislodgment on day 12 from cultures in which microglia had been continuously depleted. Overnight adherence of these cells further diminished contamination by microglia and astrocytes. Oligodendrocytes were identified by surface staining for galactocerebroside. Approximately 90% of the cells stained positive for galactocerebroside, which is not expressed on oligodendrocyte progenitors (Suzumura et al., 1984) but is present on late precursors as well as immature and mature oligodendrocytes (Mackenzie-Graham et al., 1994). Therefore, oligodendrocytes enriched from day 12 mixed cultures represent cells at various stages of differentiation. The contaminating cells were approximately 5% astrocytes and 5% microglial cells.

Preparation of Oligodendrocyte Cultures From PARP-Knockout Mice

Primary oligodendrocyte cultures were also prepared from the cerebral cortices of 2- to 8-day-old homozygous PARP-knockout mice (PARP^{-/-}) or wild-type litter mates (PARP^{+/+}), consisting of a mixed genetic background of C57/BL6 and 129SV. The generation and development of the PARP-knockout mice used in this study is described elsewhere (Wang et al., 1995). Oligodendrocytes were isolated from the brains of 2- to 8-day-old mice according to the method of Suzumura et al. (1984) with minor modifications. The brains and meninges were removed and the tissue was minced and dissociated in 0.25% trypsin. The resulting cell suspension was washed once and cells were plated into 75 cm² flasks. Cells were cultured in medium consisting of Dulbecco's modified Eagles medium (4.5 g/L glucose) supplemented with 2 mM glutamine, 10% fetal calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. Enriched cultures of oligodendrocytes were prepared by mechanical shake-off at 10 to 12 days *in vitro*. To remove contaminating cells, oligodendrocytes were incubated overnight in 75 cm² flasks. The next day, the oligodendrocytes were replated into either 96- or 12-well plates. Oligodendrocytes were identified by surface staining for galactocerebroside with approximately 70% of the cells staining positive.

Cell Treatment

Experiments were carried out on oligodendrocytes 24 h after replating. Oligodendrocytes were exposed to peroxynitrite (60–500 µM) for 20 min (PARP assay), 1 h (MTT, lactate dehydrogenase, DNA strand breakage, apoptosis assays), or 4 h (mitochondrial membrane potential (MMP), propidium iodide, apoptosis assays) at 37°C. To treat the cells, authentic peroxynitrite was diluted in phosphate-buffered saline (PBS; pH 8.3) and added to the cells in 1/10 of the volume of the wells. Control samples were treated with PBS (pH 8.3), which did not affect the final pH of the culture medium due to the small volume being added. Decomposed peroxynitrite (stored in PBS, pH 7.2, for 2 h at 37°C) was used as a control analog in all the assays. However, decomposed peroxynitrite had no effect on any of the parameters measured. In some experiments, oligodendrocytes were pretreated for 10 min with the PARP inhibitors, 3-aminobenzamide (3AB; 1 mM) and 5-iodo-6-amino-1,2-benzopyrone (INH₂BP; 100 µM) prior to peroxynitrite exposure. Although the doses of peroxynitrite used in the studies appears to be relatively high compared to those concentrations achieved *in vivo*, peroxynitrite has a very short half-life at physiological pH and temperature (Beckman et al., 1990). Therefore, the addition of a bolus of 500 µM peroxynitrite is roughly equivalent to 10 µM peroxynitrite maintained for 1 min (Beckman et al., 1994).

Measurement of Reduction of MTT to Formazan as an Indicator of Mitochondrial Respiration

The mitochondria-dependent reduction of MTT to formazan was used as an indicator of mitochondrial respiration (Zingarelli et al., 1996). Oligodendrocytes (10⁵ cell/ml) in 96-well plates were exposed to peroxynitrite for 1 h at 37°C. Cells were then incubated with MTT (0.2 mg/ml) for 60 min at 37°C and then lysed in dimethyl sulphoxide (DMSO) (100 µl/well). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD₅₅₀ using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Data are expressed as a percentage of respiration in control cultures in the absence of peroxynitrite.

Measurement of Mitochondrial Membrane Potential

Lipophilic cations such as the fluorescent dye 3,3'-dihexyl-oxacarbocyanine iodide [DiOC6(3)] are transported into mitochondria due to the negative mitochondrial membrane potential and are concentrated within the mitochondrial matrix. The mitochondrial membrane potential was quantitated by the flow cytometric analysis of DiOC6(3)-stained cells (Zamzami et al., 1995). Oligodendrocytes (10⁶ cells/ml) were seeded into 12-well plates and exposed to peroxynitrite. After 4 h peroxynitrite treatment, oligodendrocytes were stained with 40 nM DiOC6(3) for 15 min at 37°C. Then the cells were harvested, washed once with PBS, and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). Forward and side scatter were gated on the major population of normal-sized cells. Data were analyzed using CellQuest Software (Becton-Dickinson). Results are expressed as a percentage decrease in the mitochondrial membrane potential.

Lactate Dehydrogenase Release

The loss of cell membrane integrity was assessed by measuring the release of lactate dehydrogenase (LDH) from damaged cells (Pflueger et al., 1990). Oligodendrocytes (10⁵ cell/ml) in 96-well plates were exposed to peroxynitrite for 1 h at 37°C. Cell supernatant (50 µl) was then transferred to a flat-bottomed microtitre plate and 50 µl of freshly prepared lactic acid dehydrogenase substrate mixture [54 mM L-(+) lactate, 0.66 mM 2-*p*-iodophenyl-3-*p*-nitrophenyl tetrazolium chloride, 0.28 mM phenazine methosulphate, and 1.3 mM nicotinamide dinucleotide in 0.2 M Tris buffer, pH 8.2] was added to each well. Following a 5 min incubation in the dark at room temperature, the amount of LDH released was determined by measuring the OD₄₉₀ with reference to the OD₆₃₀. The percentage cytotoxicity was calculated by comparing absorbance readings to those of the maximum LDH released from oligodendrocytes following the addition of 100 µl 0.08% Triton X-100.

Determination of Cell Death by Flow Cytometric Analysis

Oligodendrocyte death was determined by measuring the cellular uptake of propidium iodide (PI). Oligodendrocytes (10^6 cell/ml) were seeded into 12-well plates. Following a 4 h incubation with peroxynitrite, cells were stained with 5 μ g/ml PI. The cells were harvested, washed once with PBS, and analyzed on a FACSCalibur flow cytometer using the standard optics for detecting PI. Data were analyzed using CellQuest Software. Cell death is expressed as the percentage of PI-positive cells.

Determination of DNA Single-Strand Breaks

The formation of strand breaks in double-stranded DNA was determined using the alkaline unwinding method (Schraufstatter et al., 1986). Oligodendrocytes (5×10^5 cells/ml) were seeded in 12-well plates. Following a 1 h incubation with peroxynitrite, cells were harvested and the amount of double-stranded DNA in the samples was determined as described previously (Zingarelli et al., 1996). Results are expressed as the % increase in DNA strand breaks.

Measurement of PARP Activity

Activation of PARP was determined by measuring the incorporation of tritiated NAD^+ into nuclear proteins (Schraufstatter et al., 1986). Oligodendrocytes (5×10^5 cells/ml) were seeded in 12-well plates. Following treatment, the culture medium was replaced with 0.5 ml of 56 mM HEPES buffer, pH 7.5, containing 28 mM KCl, 28 mM NaCl, 2 mM MgCl_2 , 0.01% digitonin, and 125 nM NAD^+ spiked with 0.25 μ Ci of [^3H] NAD^+ . Digitonin was used to permeabilize plasma membranes. The permeabilized cells were then incubated for 10 min at 37°C, and the protein that was ribosylated with [^3H] NAD^+ was precipitated with 200 μ l of 50% trichloro acetic acid (TCA). After two washes with TCA, the protein pellet was solubilized overnight in 250 μ l of 2% sodium dodecyl sulfate in 0.1 M NaOH. The radioactivity was determined using a Wallac 1450 Microbeta Plus scintillation counter (Wallac, Gaithersburg, MD). PARP activity is expressed as cpm.

Detection of Apoptotic Cell Death

Apoptotic cell death was assessed using a photometric enzyme-immunoassay to quantify histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasm of apoptotic cells. Oligodendrocytes (10^5 cell/ml) were seeded in 24-well plates and exposed to peroxynitrite for 30 min, 1 h, and 4 h at 37°C. Apoptosis was determined using a commercially available cell death detection ELISA^{PLUS} kit according to the manu-

facturer's instructions. Results are expressed as % release of nucleosomes into the cell cytoplasm compared to a positive control.

Statistical Analysis

All values here are expressed as the mean \pm standard error of the mean (SEM) of n observations, where $n > 6$. Data sets were analyzed by one-way analysis of variance (ANOVA) followed by either posthoc Dunnett's test or Tukey's test. In all cases, $P < 0.05$ was considered significant.

RESULTS

Effect of Peroxynitrite on Mitochondrial Function in Oligodendrocytes

As mitochondrial dysfunction has been proposed to represent a point of no return during cell death (Zamzami et al., 1995), we initially examined the effects of peroxynitrite on mitochondrial function in primary oligodendrocytes. Oligodendrocytes were treated with various concentrations of peroxynitrite (60–500 μ M) and the mitochondria-dependent reduction of MTT to formazan was used as an indicator of mitochondrial function. Figure 1A shows that peroxynitrite exposure caused a concentration-dependent inhibition of mitochondrial respiration in oligodendrocytes. A significant decrease in mitochondrial function was observed in cultures treated with 60 μ M peroxynitrite, and respiration rates fell to 48% of control values in oligodendrocytes exposed to the highest dose of peroxynitrite (Fig. 1A).

Mitochondrial transmembrane potential can also be used as a functional marker of mitochondrial damage, with a decrease in mitochondrial transmembrane potential having been noted in various models of cell death (Zamzami et al., 1995; Aguilar et al., 1996; Virág et al., 1998a). Mitochondrial transmembrane potential can be readily determined by measuring the transport of the fluorescent dye DiOC6(3) into the mitochondrial matrix (Zamzami et al., 1995). In addition to directly affecting mitochondrial respiration, peroxynitrite treatment dose-dependently decreased mitochondrial transmembrane potential in primary rat oligodendrocyte cultures (Fig. 1B). However, mitochondrial transmembrane potential was less sensitive than mitochondrial respiration to the effects of peroxynitrite, with 250 μ M peroxynitrite being required to produce a significant reduction in mitochondrial transmembrane potential (Fig. 1B).

Taken together, these data illustrate that exposure to peroxynitrite results in mitochondrial dysfunction in oligodendrocytes. These findings are in accordance with previous studies by Mitrovic et al. (1996), demonstrating mitochondrial damage in a murine oligodendrocyte cell line treated with a peroxynitrite-releasing compound.

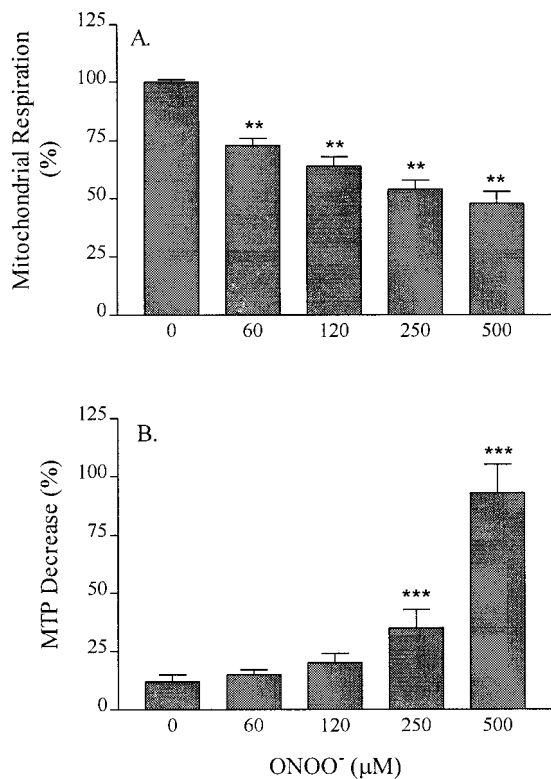


Fig. 1. Peroxynitrite (ONOO^-) induces a concentration-dependent reduction in the mitochondrial function of oligodendrocytes. **A:** Oligodendrocytes (2×10^4 cells) were exposed to native ONOO^- (60–500 μM) for 1 h and mitochondrial respiration was determined by measuring the reduction of MTT to formazan. Results are presented as % control respiration (mean \pm SEM). **B:** Oligodendrocytes (5×10^5 cells) were exposed to native ONOO^- (60–500 μM) for 4 h and mitochondrial transmembrane potential (MTP) was quantitated by the flow cytometric analysis of DiOC6(3)-stained cells. Results are expressed as % decrease in MTP (mean \pm SEM). Data were analyzed by one-way ANOVA with posthoc Dunnett's test. Double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$ compared to control group; $n = 4$ –9 from at least three independent experiments.

Peroxynitrite-Induced Oligodendrocyte Cytotoxicity

Although investigators frequently use the MTT assay as a means of assessing cell viability, under certain circumstances changes in mitochondrial respiration can be observed in the absence of cell death (Bolaños et al., 1994). Therefore, to establish whether peroxynitrite exerts additional toxic effects on oligodendrocytes, we measured LDH release from the cells following peroxynitrite exposure. Cytotoxicity is routinely assessed in vitro by measuring the release of the cytoplasmic enzyme LDH into the extracellular media (Pflueger et al., 1990). The results in Figure 2A show that peroxynitrite elicits a concentration-dependent release of LDH from oligodendrocytes. Although LDH release from peroxynitrite-treated oligodendrocytes was significantly increased from control levels ($P < 0.05$), only a small amount of LDH was elaborated compared to total values (Fig. 2A). Kim and Kim (1991) obtained similar results, with only low levels of LDH release detected

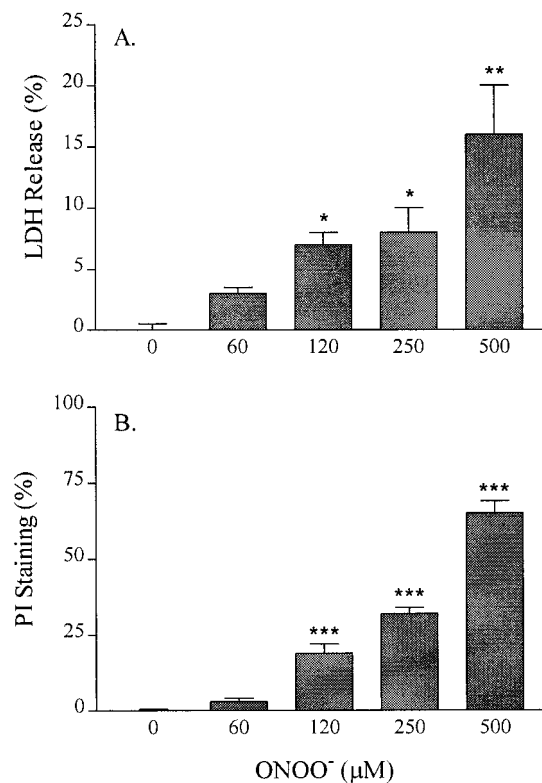


Fig. 2. Peroxynitrite (ONOO^-) exposure results in oligodendrocyte cell death. **A:** Oligodendrocytes (2×10^4 cells) were exposed to native ONOO^- (60–500 μM) for 1 h and cell membrane injury was quantitatively assessed by measuring the release of LDH from damaged and dying cells. Data are presented as % total cell lysis achieved by the addition of Triton-X (mean \pm SEM). **B:** Oligodendrocytes (5×10^5 cells) were exposed to native ONOO^- (60–500 μM) for 4 h and cell death was determined by the flow cytometric measurement of PI uptake. Results are expressed as % increase in PI uptake (mean \pm SEM). Data were analyzed by one-way ANOVA with posthoc Dunnett's test. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$ compared to control group; $n = 4$ –12 from at least three independent experiments.

after exposure of bovine oligodendrocytes to oxygen radicals. Moreover, Mitrovic et al. (1996) have suggested that as oligodendrocytes have lipid-rich membranes, they do not liberate LDH when exposed to peroxynitrite-releasing compounds. Instead, the increased amounts of LDH released from our cultures may be a consequence of the toxic effects of peroxynitrite on contaminating cells such as astrocytes. Therefore, in order to confirm whether peroxynitrite exposure induces lethal effects in oligodendrocytes, cell death was also determined by measuring the cellular uptake of PI, a fluorescent substance that is normally excluded by intact cells. Exposure of oligodendrocytes to peroxynitrite resulted in dose-dependent cytotoxicity as demonstrated by an increase in PI uptake (Fig. 2B). A significant increase in PI uptake was observed in oligodendrocytes treated with 120 μM peroxynitrite, with 65% of the cells staining positive for PI in cultures exposed to the highest dose of peroxynitrite (Fig. 2B). Previous studies have demonstrated that 50 μM to 1 mM peroxynitrite exerts toxic effects on CNS cells

(Cookson et al., 1998; Endres et al., 1998; Cassina et al., 2002). However, the question arises as to whether the concentrations of peroxynitrite used in such studies are physiologically relevant. Stimulated macrophages generate peroxynitrite at the rate of 0.1 nmol/10⁶ cells/min, which translates into rates of peroxynitrite formation in the range of 7 μ M/min within a blood vessel (Ischiropoulos et al., 1992a). Moreover, cells that are in direct contact with immunostimulated cells, as would occur in MS and spinal cord injury, are likely exposed to even higher levels of peroxynitrite. As peroxynitrite has a very short half-life in vitro (Beckman et al., 1990), the addition of a bolus of 500 μ M peroxynitrite equates to approximately 10 μ M peroxynitrite maintained for 1 min (Beckman et al., 1994). Therefore, our results verify that pathophysiologically relevant concentrations of peroxynitrite are toxic to primary rat oligodendrocytes.

Formation of DNA Strand Breaks and Activation of PARP in Peroxynitrite-Treated Oligodendrocytes

In other cellular systems, peroxynitrite has been shown to mediate toxicity indirectly through activation of PARP (Szabó, 1996; Endres et al., 1998; Virág et al., 1998c). We therefore assessed whether PARP is activated in oligodendrocytes following peroxynitrite treatment. Peroxynitrite triggers DNA strand breakage and the subsequent activation of PARP (Szabó, 1996). We examined the ability of peroxynitrite to damage DNA in oligodendrocytes by measuring the formation of DNA strand breaks. As shown in Figure 3A, peroxynitrite induced significant DNA strand breakage in oligodendrocytes ($P < 0.001$). Activation of PARP was then determined by measuring the incorporation of tritiated NAD⁺ into oligodendrocyte nuclear proteins (Schraufstatter et al., 1986). Figure 3B demonstrates that while oligodendrocytes exhibit a low level of basal enzyme activity, exposure to peroxynitrite greatly augmented PARP activity. Furthermore, treatment with 500 μ M peroxynitrite induced approximately a three-fold increase in enzyme activity (Fig. 3B).

Effect of PARP Inhibitors on Peroxynitrite-Induced Oligodendrocyte Cytotoxicity

As significant PARP activation was observed in oligodendrocytes following peroxynitrite treatment, we investigated whether the toxic effects of peroxynitrite on oligodendrocytes were mediated via activation of this enzyme. Previous studies have established that pharmacological inhibitors of PARP are effective in preventing peroxynitrite-induced toxicity in a variety of cell types, including astrocytes and thymocytes (Endres et al., 1998; Virág et al., 1998a, 1998b). In the following experiments, oligodendrocyte cultures were treated with either of two structurally unrelated PARP

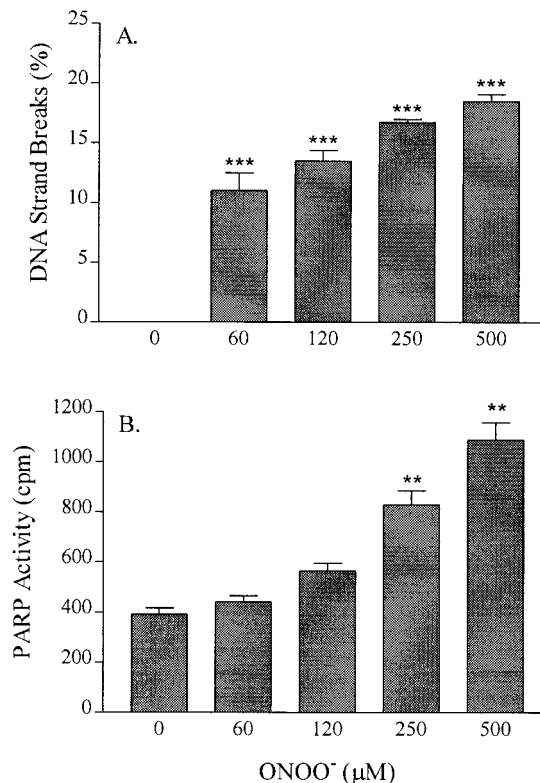


Fig. 3. DNA strand breakage and activation of PARP in oligodendrocytes treated with peroxynitrite (ONOO⁻). **A:** Oligodendrocytes (5×10^5 cells) were exposed to native ONOO⁻ (60–500 μ M) for 1 h and DNA strand breaks were measured by the alkaline unwinding method. Data are presented as % increase in DNA strand breaks compared to control cells. **B:** Oligodendrocytes (10^6 cells) were exposed to native ONOO⁻ (60–500 μ M) for 20 min and activation of PARP was determined by measuring the incorporation of tritiated NAD⁺ into nuclear proteins. Results are expressed as cpm (mean \pm SEM). Data were analyzed by one-way ANOVA with posthoc Dunnett's test. Double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$ compared to control group; $n = 4-6$ from at least two independent experiments.

inhibitors, 3AB (1 mM) and INH₂BP (100 μ M), prior to peroxynitrite exposure. Parameters of cell toxicity including mitochondrial dysfunction, LDH release, and PI staining were then redetermined (Fig. 4). PARP activity was also assessed to confirm that 3AB and INH₂BP, at the doses used, effectively inhibited enzyme activity in oligodendrocytes. Figure 5 shows that the presence of both 3AB and INH₂BP prevented PARP activation in oligodendrocytes in response to peroxynitrite, with 63% and 72% inhibition of PARP, respectively. However, pretreatment with 3AB or INH₂BP did not protect oligodendrocytes from the toxic actions of peroxynitrite (Fig. 4). Therefore, peroxynitrite evidently mediates oligodendrocyte toxicity independently of the activity of PARP.

Peroxynitrite-Induced Toxicity in Oligodendrocytes From PARP^{-/-} Mice

The failure of the 3AB and INH₂BP to prevent peroxynitrite-induced toxicity may be due to the lack of

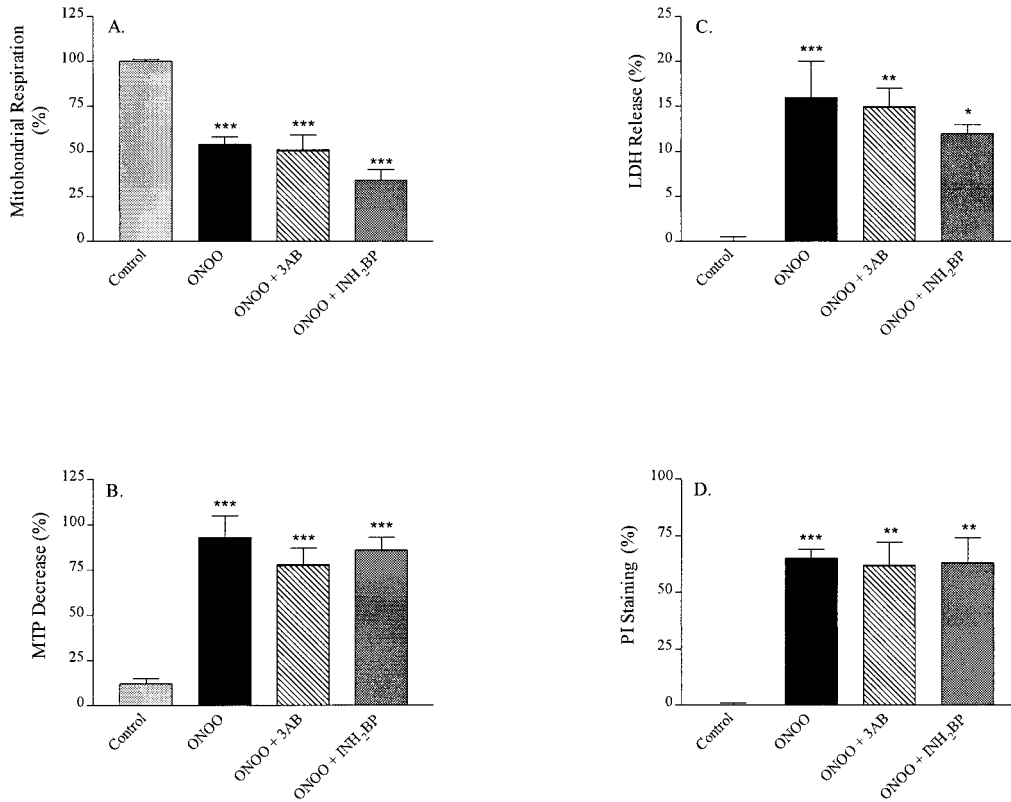


Fig. 4. PARP inhibitors do not protect oligodendrocytes from the cytotoxic effects of peroxynitrite (ONOO⁻). Oligodendrocytes were pretreated with either 1 mM 3AB or 100 μM INH₂BP for 10 min and then exposed to 500 μM native ONOO⁻. **A:** Mitochondrial respiration was determined 1 h later by measuring the reduction of MTT to formazan. Results are presented as % control respiration (mean ± SEM). **B:** MTP was quantitated 4 h later by the flow cytometric analysis of DiOC6(3)-stained cells. Results are expressed as % decrease in MTP (mean ± SEM). **C:** Cell membrane injury was quanti-

tatively assessed 1 h later by measuring the release of LDH from damaged and dying cells. Data are presented as % total cell lysis achieved by the addition of Triton-X (mean ± SEM). **D:** Cell death was determined 4 h later by the flow cytometric measurement of PI uptake. Results are expressed as % increase in PI uptake (mean ± SEM). Data were analyzed by one-way ANOVA with posthoc Tukey's test. Asterisk, *P* < 0.05; double asterisk, *P* < 0.01; triple asterisk, *P* < 0.001 compared to control group; n = 4–6 from at least three independent experiments.

specificity and potency of these PARP inhibitors. Therefore, to confirm that peroxynitrite does induce oligodendrocyte death independently of PARP activation, we examined the effects of peroxynitrite on oligodendrocyte cultures generated from homozygous PARP-knockout mice (PARP^{-/-}; Fig. 6). Oligodendrocytes from PARP^{-/-} animals or wild-type litter mates (PARP^{+/+}) were treated with various concentrations of peroxynitrite and mitochondrial dysfunction and LDH release were assessed (Fig. 6). PARP activity was also determined to verify that PARP^{-/-} oligodendrocytes lack specific enzyme activity (Fig. 7). Figure 6A shows that peroxynitrite exposure caused a concentration-dependent inhibition of mitochondrial respiration in both PARP^{+/+} and PARP^{-/-} oligodendrocytes. Similarly, peroxynitrite elicited a dose-dependent release of LDH from PARP^{+/+} and PARP^{-/-} oligodendrocytes (Fig. 6B). Although the deleterious actions of peroxynitrite were slightly diminished in PARP^{-/-} cells, there was no significant difference in the sensitivity of the PARP^{+/+} and PARP^{-/-} oligodendrocytes to peroxynitrite-induced toxicity. In contrast, peroxynitrite exposure significantly increased PARP activity in PARP^{+/+}

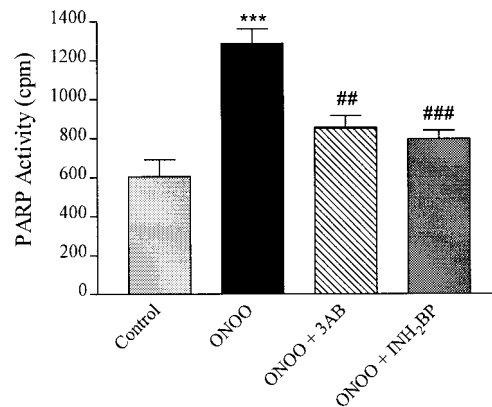


Fig. 5. PARP inhibitors prevent PARP activation in peroxynitrite (ONOO⁻)-treated oligodendrocytes. Oligodendrocytes (10⁶) were pretreated with either 1 mM 3AB or 100 μM INH₂BP for 10 min and then exposed to 500 μM native ONOO⁻. Activation of PARP was determined after 20 min by measuring the incorporation of tritiated NAD⁺ into nuclear proteins. Results are expressed as cpm (mean ± SEM). Data were analyzed by one-way ANOVA with posthoc Tukey's test. Triple asterisk, *P* < 0.001 compared to control group; double number sign, *P* < 0.01; triple number sign, *P* < 0.001 compared to ONOO⁻ group; n = 6–12 from at least three independent experiments.

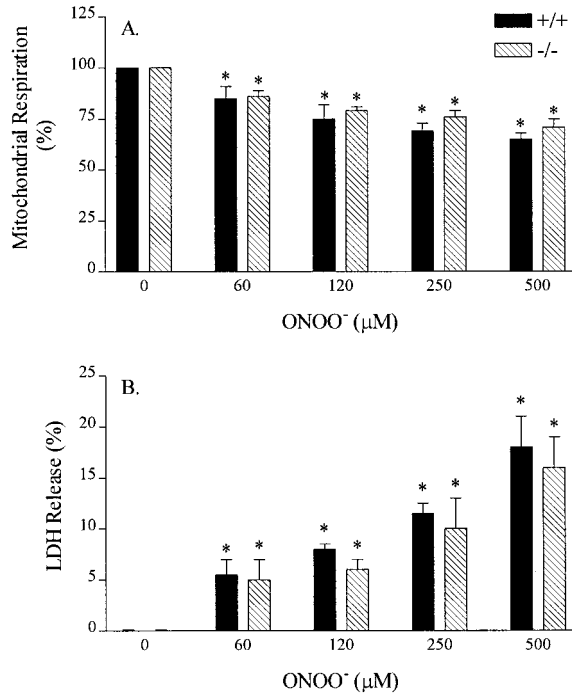


Fig. 6. Peroxynitrite (ONOO⁻) induces concentration-dependent cytotoxic effects on primary oligodendrocytes isolated from PARP^{-/-} and PARP^{+/+} mice. **A:** Oligodendrocytes (2×10^4 cells) were exposed to native ONOO⁻ (60–500 μM) for 1 h and mitochondrial respiration was determined by measuring the reduction of MTT to formazan. Results are presented as % control respiration (mean \pm SEM). **B:** Oligodendrocytes (2×10^4 cells) were exposed to native ONOO⁻ (60–500 μM) for 1 h and cell membrane injury was quantitatively assessed by measuring the release of LDH from damaged and dying cells. Data are presented as % total cell lysis achieved by the addition of Triton-X (mean \pm SEM). Data were analyzed by one-way ANOVA with posthoc Dunnett's test. Asterisk, $P < 0.01$ compared to control group; $n = 6$ from at least three independent experiments.

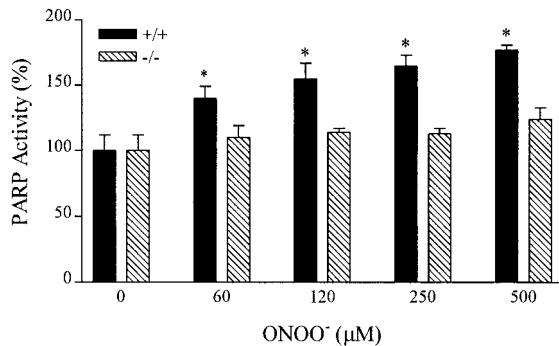


Fig. 7. Activation of PARP in PARP^{-/-} and PARP^{+/+} oligodendrocytes treated with peroxynitrite (ONOO⁻). Oligodendrocytes (10^6 cells) were exposed to native ONOO⁻ (60–500 μM) for 20 min and activation of PARP was determined by measuring the incorporation of tritiated NAD⁺ into nuclear proteins. Results are expressed as % control activity (mean \pm SEM). Data were analyzed by one-way ANOVA with posthoc Dunnett's test. Asterisk, $P < 0.05$ compared to control group; $n = 6$ from at least three independent experiments.

oligodendrocytes but did not induce enzyme activity in PARP^{-/-} cells (Fig. 7). These results corroborate our findings using PARP inhibitors by demonstrating that

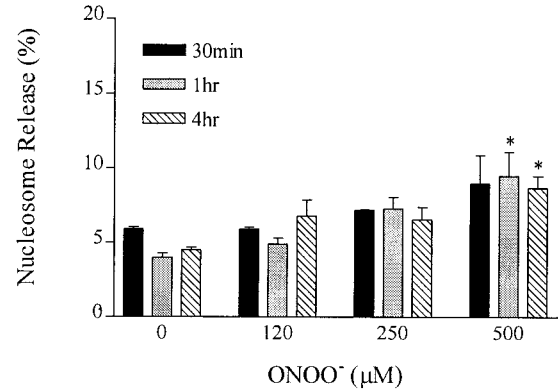


Fig. 8. Apoptosis is minimal in primary rat oligodendrocytes exposed to peroxynitrite (ONOO⁻). Oligodendrocytes (2×10^4 cells) were exposed to native ONOO⁻ (120–500 μM) for 30 min, 1 h, and 4 h and apoptosis was determined using an ELISA to quantify the release of mono- and oligonucleosomes into the cell cytoplasm. Results are presented as % increase in nucleosome release compared to the positive control (mean \pm SEM). Data were analyzed by one-way ANOVA with posthoc Dunnett's test. Asterisk, $P < 0.05$ compared to control group; $n = 4–6$ from at least three independent experiments.

peroxynitrite-induced oligodendrocyte toxicity occurs independently of PARP activation.

Peroxynitrite-Induces Minimal Apoptosis in Primary Cultures of Rat Oligodendrocytes

While we have confirmed that peroxynitrite is cytotoxic for primary oligodendrocytes, we have not established whether cell death occurs by necrosis or apoptosis. Oligodendrocytes have been reported to undergo necrosis and apoptosis *in vitro* in response to NO and hydrogen peroxide, respectively (Mitrovic et al., 1995; Richter-Landsburg and Vollgraf, 1998; Volgraff et al., 1999; Brand et al., 2001). It is known that PARP inhibitors do not protect thymocytes from apoptotic cell death (Virág et al., 1998c). Therefore, it is possible that 3AB and INH₂BP did not inhibit cytotoxicity in our cultures because peroxynitrite-induced oligodendrocyte cell death was mediated via apoptosis. To determine whether this was indeed the case, we used a sensitive ELISA method to detect apoptosis in oligodendrocyte cultures exposed to peroxynitrite. The ELISA quantitatively assesses the release of mono- and oligonucleosomes into the cytoplasm of apoptotic cells. A small number of nucleosomes were detected in the cytoplasm of control-treated oligodendrocyte cultures, indicating that some cells were undergoing apoptosis (Fig. 8). This is to be expected as apoptotic cell death occurs naturally. Figure 8 shows that apoptosis was not markedly increased in oligodendrocytes treated with 125 and 250 μM peroxynitrite even after 4 h of exposure (Fig. 8). However, significantly more nucleosomes were released by oligodendrocyte cultures exposed to 500 μM peroxynitrite for 1 and 4 h ($P < 0.05$). Nevertheless, only minimal apoptosis was observed in these cultures (Fig. 8). Our findings suggest

that peroxynitrite does not induce marked oligodendrocyte apoptosis. Instead, oligodendrocytes largely undergo necrotic cell death following exposure to peroxynitrite.

DISCUSSION

Oligodendrocyte loss is a characteristic feature of several CNS conditions, including MS and spinal cord injury. However, the precise mechanisms through which oligodendrocytes are destroyed remain unknown. Recent studies have implicated peroxynitrite in the pathogenesis of both spinal cord injury and MS (Hooper et al., 1997; Cross et al., 1998; Scott et al., 1999; Liu et al., 2000; Xu et al., 2001). Furthermore, peroxynitrite has been shown to induce axonal damage and demyelination *in vivo* (Touil et al., 2001) and deleterious effects of peroxynitrite-releasing compounds on an oligodendrocyte cell line have been reported (Mitrovic et al., 1996). Here we have assessed the nature of peroxynitrite-mediated toxic effect on primary oligodendrocytes. The current study clearly demonstrates that peroxynitrite exerts toxic effects on primary oligodendrocytes and may therefore be responsible for the oligodendrocyte damage and demyelination observed in many CNS disorders.

Peroxyntirite is capable of inducing cellular injury indirectly by triggering DNA single-strand breakage and activating PARP (Szabó, 1996). Activation of PARP can rapidly deplete intracellular levels of NAD^+ , slowing the rate of glycolysis, electron transport, and ATP formation, resulting in cell dysfunction and death (Szabó, 1998). PARP activation has previously been associated with CNS cell death (Endres et al., 1998; Ha and Snyder, 2000; Lee et al., 2001). Moreover, pharmacological inhibitors of PARP are known to protect against cell damage in response to exogenously produced peroxynitrite (Cookson et al., 1998; Endres et al., 1998; Virág et al., 1998b, 1998c). Once we had ascertained that peroxynitrite induced oligodendrocyte toxicity, we then went on to assess whether activation of PARP was involved in mediating oligodendrocyte death following peroxynitrite exposure. DNA damage and PARP activity were significantly increased in primary oligodendrocytes exposed to peroxynitrite, suggesting that activation of PARP may play a role in oligodendrocyte toxicity. However, in our study, the presence of the PARP inhibitors 3AB and INH_2BP in the culture system failed to protect oligodendrocytes from peroxynitrite-induced cytotoxicity. Similarly, Groit et al. (1990) demonstrated that PARP inhibitors did not prevent superoxide-induced death in primary dog oligodendrocytes. Moreover, we have shown that peroxynitrite exerts toxic effects on primary oligodendrocytes isolated from PARP-knockout mice. These observations indicate that oligodendrocyte cell death, in response to peroxynitrite and reactive oxygen species, occurs independently of PARP activation. In addition, other investigators have shown that PARP activation following

oxidative stress did not contribute to cell death in primary hepatocyte cultures (Yamamoto et al., 1993; Lator et al., 2000). Yamamoto et al. (1993) proposed that cytotoxicity following PARP activation may be dependent on the cell type. More specifically, nonproliferating cells such as hepatocytes likely have a different outcome with respect to oxidative damage to DNA than proliferating cells. Activation of PARP in proliferating cells is required to repair any damage to DNA, as single-strand breaks prevent DNA replication. On the other hand, DNA replication does not occur in resting cells and therefore there is little requirement for PARP activity. Thus, it is conceivable that PARP overactivation following oxidative stress only induces death in proliferating cells due to differences in both the location and level of enzyme activity in both cell types. This hypothesis could help explain why inhibiting PARP activity had no effect on peroxynitrite-induced death of primary oligodendrocytes as the culture conditions used in our study may not be conducive to oligodendrocyte cell division (Suzumura et al., 1984). Similarly, it is well known that mature oligodendrocytes do not proliferate (Verity et al., 1993).

As well as mediating cell death via PARP activation, peroxynitrite may induce apoptosis (Estevez et al., 1995; Lin et al., 1995; Virág et al., 1998a, 1998c). At present, there is some debate as to whether oligodendrocytes undergo apoptotic cell death (Merrill and Scolding, 1999). However, *in vivo* evidence exists demonstrating that oligodendrocyte apoptosis occurs during MS and spinal cord injury (Lucchinetti et al., 1994; Ozawa et al., 1994; Shuman et al., 1997; Casha et al., 2001). Furthermore, oligodendrocytes undergo apoptosis *in vitro* following exposure to hydrogen peroxide (Richter-Landsburg and Vollgraf, 1998; Vollgraf et al., 1999; Brand et al., 2001). As it is known that PARP inhibitors are unable to protect thymocytes from apoptotic death (Virág et al., 1998), it is therefore conceivable that 3AB and INH_2BP had no effect on peroxynitrite-induced cytotoxicity in oligodendrocytes because this is mediated through a PARP-independent apoptotic pathway. However, this does not appear to be the case as our findings indicate that peroxynitrite mediates oligodendrocyte cell death primarily via necrosis rather than apoptosis.

While activation of PARP does not appear to mediate oligodendrocyte toxicity following peroxynitrite exposure, enzyme activity is markedly increased in these cells. In contrast to its role in cell death, PARP has also been shown to be involved in cell survival (Bürkle, 2001). PARP has been implicated in the maintenance of genomic stability (Masutani et al., 2000) as well as in the regulation of gene transcription and proteosomal function (Ullrich et al., 1999; Kameoka et al., 2000). Moreover, Vispé et al. (2000) have recently identified a novel cellular defense pathway, mediated by PARP, that regulates transcription in response to DNA damage. Thus, PARP may be activated in peroxynitrite-treated oligodendrocytes as a protective mechanism. In

this context, inhibition of PARP would have a deleterious effect on cell survival.

The data presented in this study clearly demonstrate that primary oligodendrocytes are sensitive to the cytotoxic effects of peroxynitrite, although cell death appears to be independent of PARP activation. However, peroxynitrite is capable of mediating a variety of other potentially toxic interactions, including nitration of tyrosine residues (Ischiropoulos et al., 1992b). Tyrosine nitration has been shown to interfere with protein function (Szabó, 1996). Furthermore, as phosphorylation of protein tyrosine residues plays a major role in regulating a number of metabolic pathways, the formation of nitrotyrosine may negatively influence cellular function (Nakazawa et al., 2000). Nitration of tyrosine residues in oligodendrocyte proteins by peroxynitrite could potentially lead to cellular dysfunction and death. Interestingly, nitrotyrosine formation has been observed in CNS tissues from MS patients (Bagasra et al., 1995; Hooper et al., 1997; Cross et al., 1998; Liu et al., 2001), EAE-diseased animals (Cross et al., 1997; Van der Veen et al., 1997; Hooper et al., 2000; Scott et al., 2001), and spinal cord-injured rats (Scott et al., 1999; Xu et al., 2001). Moreover, in spinal cord-injured tissues nitrotyrosine residues have been associated with areas of active cell death (Scott et al., 1999), while in MS brains nitrotyrosine has been detected in myelin membranes (Liu et al., 2001).

Peroxyntirite-mediated lipid peroxidation is another candidate for the mechanism of oligodendrocyte cell death. It is noteworthy in this regard that increased lipid peroxidation occurs in a variety of neuropathological conditions, including spinal cord injury and MS (Braugher and Hall, 1992; Toshniwal and Zarling, 1992). Moreover, administration of lipid peroxidation inhibitors promotes functional recovery in animal models of spinal cord injury (Diaz-Ruiz et al., 1999). Oligodendrocytes are likely candidates for injury triggered by lipid peroxidation due to the high lipid content of their membranes (Mitrovic et al., 1996). Moreover, the lipid peroxidation product, 4-hydroxynonenal, has been shown to be toxic to oligodendrocytes in vitro (McCrahen et al., 2000). Lipid peroxidation can result in cell death by interfering with mitochondrial function or altering signaling cascades (Keller and Mattson, 1998). As there is evidence that peroxynitrite can peroxidate purified myelin (van der Veen and Roberts, 1999), it is highly plausible that peroxynitrite induces oligodendrocyte cytotoxicity through lipid peroxidation.

Another mechanism through which peroxynitrite may exert cytotoxic effects is by directly inhibiting mitochondrial respiration, thus leading to energetic failure and cell death (Boczkowski et al., 2001). Mitochondrial damage has been suggested to be an important event in the pathogenesis of a range of neurological disorders (Heales et al., 1999). Previous work has also shown that peroxynitrite inhibited complexes of the mitochondrial respiratory chain in neurons (Bolaños et al., 1995). Furthermore, this damage preceded cell death, suggesting that mitochondrial dysfunction

may be responsible for peroxynitrite-induced neurotoxicity (Bolaños et al., 1995). Here we have verified that there is a loss of mitochondrial function in oligodendrocytes exposed to peroxynitrite. In addition, we have demonstrated that the mitochondrial membrane potential decreases in oligodendrocytes following peroxynitrite treatment, indicating that the mitochondria have undergone a permeability transition. It remains to be established whether peroxynitrite-mediated mitochondrial damage directly causes oligodendrocyte death in MS and spinal cord injury. Nevertheless, our studies have associated a peroxynitrite-induced loss of mitochondrial function in primary oligodendrocytes with evidence of cell death.

In summary, we have reported that peroxynitrite can cause toxicity in primary oligodendrocytes. Since the peroxynitrite-mediated oligodendrocyte death is not dependent on PARP activity, peroxynitrite exerts cytotoxic effects on oligodendrocytes through other mechanisms. Potential pathogenic pathways include tyrosine nitration and lipid peroxidation. However, we have provided evidence to suggest that peroxynitrite induces oligodendrocyte death by interfering with mitochondrial function. Our results imply that the oligodendrocyte loss and demyelination observed in MS and spinal cord injury may be due to the toxic effects of peroxynitrite on oligodendrocytes. Therefore, the use of pharmacological agents that neutralize or interfere with the generation of peroxynitrite may be useful therapeutics in the treatment of these conditions.

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