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The heme precursor delta-aminolevulinate blocks peripheral myelin formation

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Abstract

Delta-aminolevulinic acid (δ -ALA) is a heme precursor implicated in neurological complications associated with porphyria and tyrosinemia type I. Delta-ALA is also elevated in the urine of animals and patients treated with the investigational drug dichloroacetate (DCA). We postulated that δ -ALA may be responsible, in part, for the peripheral neuropathy observed in subjects receiving DCA. To test this hypothesis, myelinating cocultures of Schwann cells and sensory neurons were exposed to δ -ALA (0.1–1 mM) and analyzed for the expression of neural proteins and lipids and markers of oxidative stress. Exposure of myelinating samples to δ -ALA is associated with a pronounced reduction in the levels of myelin-associated lipids and proteins, including myelin protein zero and peripheral myelin protein 22. We also observed an increase in protein carbonylation and the formation of hydroxynonenal and malondialdehyde after treatment with δ -ALA. Studies of isolated Schwann cells and neurons indicate that glial cells are more vulnerable to this pro-oxidant than neurons, based on a selective decrease in the expression of mitochondrial respiratory chain proteins in glial, but not in neuronal, cells. These results suggest that the neuropathic effects of δ -ALA are attributable, at least in part, to its pro-oxidant properties which damage myelinating Schwann cells.

Six keywords

Schwann cells; demyelination; delta-aminolevulinate; peripheral neuropathy; oxidative stress; dichloroacetate

Introduction

Delta-aminolevulinic acid (δ -ALA) is an early precursor in heme synthesis (Monteiro et al. 1986). Urinary concentrations of δ -ALA are elevated in hereditary porphyrias (Becker and Kramer 1977; Gorchein and Webber 1987), hereditary tyrosinemia type I (Mitchell et al. 1990) and in industrial workers exposed to heavy metals, especially lead (Sakai and Morita 1996; Sakai 2000). Urinary excretion of δ -ALA is also increased in children with genetic mitochondrial diseases who are treated with the investigational drug dichloroacetate (DCA)

(Stacpoole et al. 2006). This effect is attributed to the inhibition of maleylacetoacetate isomerase, the penultimate enzyme in the tyrosine catabolic pathway that also functions as glutathione transferase to dechlorinate DCA. The block in distal tyrosine metabolism in individuals chronically exposed to DCA, or who have tyrosinemia type I, is thought to divert tyrosine-derived carbon toward succinylacetone formation (Ammini et al. 2003). Succinylacetone inhibits δ -ALA dehydratase (porphobilinogen synthase) and causes accumulation of δ -ALA. The peripheral neuropathy that occurs in some patients with tyrosinemia type I (Russo et al. 2001) has been attributed to an increase in tissue concentrations of δ -ALA per se and/or to the inhibition of heme synthesis. Rats and humans chronically exposed to DCA at doses of 25 mg/kg/d or higher may also develop a reversible sensory-motor peripheral neuropathy that is associated with increased urinary levels of δ -ALA (Stacpoole 1989; Stacpoole et al., 1998; Moser et al., 1999; Spruijt et al., 2001; Kaufmann et al., 2006; Stacpoole et al., 2006).

It is uncertain whether elevated levels of δ -ALA in these conditions are merely a surrogate marker of disease (Gorchein and Webber 1987; Sakai and Morita 1996; Lindberg et al. 1999) or contribute directly to the pathophysiological manifestations of neuropathy (Demasi et al. 1996; Adhikari et al. 2006). Delta-ALA is a structural analog of gamma-aminobutyric acid (GABA) and interacts with GABA receptors (Demasi et al. 1996). It is also a pro-oxidant because of the presence of a potentially reactive keto group on the molecule, and this property has been implicated in δ -ALA-induced damage to GABA receptors and to mitochondria (Monteiro et al. 1986; Hermes-Lima et al. 1992; Vercesi et al. 1994; Demasi et al. 1996; Princ et al. 1998; Adhikari et al. 2006). Consistent with this mechanism are the findings that CNS and liver toxicity associated with δ -ALA exposure are reduced by coadministration of the antioxidants melatonin and bilirubin (Princ et al. 1997; Carneiro and Reiter 1998; Noriega et al. 2003).

Myelin appears to be particularly susceptible to oxidative damage, as suggested by studies of demyelinating disorders and the possible role of reactive oxygen and nitrogen species in their pathogenesis (Smith et al. 1999). In the CNS this may partially reflect the high energy requirement (Bondy and LeBell 1991) and high iron content (Bartzokis 2004) of oligodendrocytes, in addition to their relatively low levels of glutathione (GSH) (Juurlink et al. 1998). These conditions may lead to a free radical-induced insult to the brain, which likely would be mediated by increased levels of peroxides (LeBel and Bondy 1991). All peroxides are scavenged by glutathione peroxidase. Glutathione peroxidase activity requires GSH as an electron donor, resulting in the formation of oxidized glutathione. The combination of high energy requirement and low levels of GSH suggests that oligodendrocytes have a poor ability to scavenge peroxides. Indeed, oligodendrocytes are highly vulnerable to oxidative stress associated with ischemia (Pantoni et al. 1996). Similarly, Schwann cells (SCs) are susceptible to oxidative stress under conditions of ischemia-reperfusion injury (Ida et al., 2004) and diabetic neuropathy (Wada and Yagihashi 2005). The GSH levels in sciatic nerves (Romero et al. 1991) and the GSH-related specific activities (glutathione reductase, peroxidase and transferase) associated with the cytosolic fraction (Romero et al. 1990) are very low, as compared to brain and liver, respectively. Together, these studies suggest that myelinating glial cells are susceptible to damage by pro-oxidant compounds.

Because of the known deleterious effects of δ -ALA exposure on the nervous system and its apparent accumulation in several congenital and acquired conditions, we investigated its effect on primary cultures of rat SCs and peripheral sensory neurons. Our results indicate that δ -ALA exposure has a striking negative effect on myelinating glial cells, both during the active phase of myelination and myelin maintenance. These effects are likely mediated by the pro-oxidant properties of δ -ALA.

Materials and methods

Antibodies

Table 1 lists the primary antibodies used in this study. Secondary antibodies for Western blots were anti-mouse and anti-rabbit horseradish peroxidase conjugate (Cell Signaling Technology®, Inc., Beverly, MA), both at 1:5,000 dilution, and anti-rat (Sigma, St. Louis, MO) horseradish peroxidase conjugate at 1:1,000. We used Alexa Fluor 488 (green) anti-mouse or anti-rat (Molecular Probes, Invitrogen Corporation, Carlsbad, CA) at 1:500 dilution for the immunocytochemical studies.

Delta-aminolevulinate

A stock solution of 100 mM δ -ALA hydrochloride (Sigma, St. Louis, MO) was prepared in 80% Minimum Essential Medium (Gibco®, Invitrogen Corporation, Carlsbad, CA) and 20% one molar HEPES (Cellgro, Mediatech, Inc., Herndon, VA), filtered through 0.22 μ m syringe filter (Fisher Scientific, Pittsburg, PA) and stored in aliquots at -20°C . The solution was diluted in the appropriate culture medium to which δ -ALA was added at a final concentration of 0.1–5.0 mM. Addition of δ -ALA did not appreciably change the final pH of the medium. Each aliquot of the stock solution was used once.

Cell culture models

Primary rat Schwann cells—Rat Schwann cell (SC) cultures were established from neonatal sciatic nerves as described (Weinmaster and Lemke 1990). Nerves were enzymatically treated with collagenase and trypsin, followed by mechanical dissociation. The cell suspension was filtered, and cells were plated on tissue culture plastic in Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, Invitrogen Corporation, Carlsbad, CA) with 10% fetal calf serum (FCS) (Hyclone, Logan, UT). For the next 4 days, the culture medium contained 10 μ M cytosine arabinoside to decrease fibroblast proliferation. SCs were grown in normal growth medium (DMEM/10% FCS, 2 μ M forskolin, and 10 μ g/mL glial growth factor) on poly-L-lysine-coated culture dishes or glass coverslips. Exposure of cells to δ -ALA commenced the next day after plating and continued for 72 hours, or as indicated.

Dorsal root ganglion neurons—Dissociated neuronal cultures from rats were established as described (Notterpek et al. 1999). Dorsal root ganglia (DRG) were collected from embryonic day 15 rodents, digested with 0.25% trypsin (Gibco, Rockville, MD), mechanically dissociated and plated on rat tail collagen (Biomedical Technologies, Inc., Stoughton, MA) coated 12 mm glass coverslips for immunolabeling and on collagen coated tissue culture plastic for biochemical studies and toxicology assays. Cultures were maintained in Minimum Essential Medium (Gibco®, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% FCS, 0.3% glucose (Sigma-Aldrich, St. Louis, MO), 10 mM HEPES and 100 ng/mL nerve growth factor (Harlan Bioproducts for Science, Madison, WI). The day after plating, the cultures were treated with fluorodeoxyuridine (10 μ M) for three cycles to enrich the neuronal population. A six-day δ -ALA exposure (0.1–1.0 mM) was initiated one week after the last fluorodeoxyuridine treatment. At the end of δ -ALA treatment, the cultures were used for immunolabeling or biochemical assays.

Myelinating cocultures of SCs-DRG neurons—Myelinating cocultures of SCs-DRG neurons were established as described (Einheber et al. 1993). SCs were added to DRG cultures one week after the third fluorodeoxyuridine cycle and were allowed to proliferate for 10–12 days. To initiate myelination, the medium was supplemented with ascorbic acid (50 μ g/mL; Sigma-Aldrich, St. Louis, MO). For the dysmyelination studies, the beginning of δ -ALA treatment coincided with the initiation of myelin formation and continued for 10 days. Control and δ -ALA-exposed cultures were then used for immunolabeling and

biochemical experiments. For the demyelination studies, cocultures were allowed to myelinate for 12 days, and subsequently, the medium was supplemented with δ -ALA for an additional 12 days. Samples were then processed for biochemical experiments.

Ex vivo δ -ALA treatment of myelinated peripheral nerves

Sciatic nerves were isolated from 9 month-old mice and immersed into Minimum Essential Medium, with or without supplementation with 5 mM δ -ALA. Nerves were teased and incubated at 37 °C and 5% CO₂ for four hours and then homogenized in SDS gel sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 3% SDS) supplemented with protease and phosphatase inhibitors. Lysates were probed by Western blots for changes in the levels of protein carbonyls, hydroxynonenal (HNE), and malondialdehyde (MDA).

Immunocytochemistry

Control and δ -ALA treated DRG neurons or cocultures were fixed with 4% paraformaldehyde for 10 min, permeabilized with 100% methanol at -20 °C for 5 min and blocked with 10% normal goat serum in phosphate buffered saline for 1 hour. Cells were incubated overnight at 4 °C with primary antibodies, and bound antibodies were detected using Alexa Fluor conjugates. Hoechst dye was included with the secondary antibodies to visualize nuclei. Control samples without specific primary antibodies were processed in parallel. Cover-slips were mounted using the Prolong Antifade kit (Molecular Probes, Invitrogen Corporation, Carlsbad, CA) and images were acquired by a Leica TCS SP2 AOBS Spectral Confocal Microscope. Scans were obtained every 0.5 μ m, and the most intense layer was used. Images were processed with Leica Confocal Software Version 2.61, Build 1537, and figures were assembled using Adobe Photoshop 5.5.

Mitochondrial polarity assay

SCs that had been in culture for three days were washed with DMEM twice to remove serum and maintained in serum free medium for three hours with or without the addition of 5 mM δ -ALA. They were agitated for 15 minutes in the presence of 10 μ M of JC-1 mitochondrial polarity dye (Molecular Probes®, Invitrogen, Carlsbad, CA) and rinsed twice with DMEM. After trypsinization, the cells were suspended in 10% FCS in DMEM and analyzed by flow cytometry for orange (590 nm) and green (525 nm) fluorescence. Cell localization on orange/green fluorescence map was compared with that of cells treated with 100 nM of valinomycin, which is known to depolarize mitochondrial membranes (Morrison et al. 2005). A shift from orange to green emission is indicative of mitochondrial depolarization (Reers et al. 1991).

Mass spectrometric analysis of myelin lipids

1,2-didodecanoyl-*rac*-glycero-3-phosphocholine (>99%) was purchased from Sigma (St Louis, MO) to be used as an internal standard for mass spectrometry. Isopropyl alcohol (HPLC grade) was purchased from Fisher Scientific (Pittsburg, PA). All other chemicals were reagent grade or better. Control and δ -ALA exposed samples were treated with 10% HCl, homogenized using conical pestles for 5 to 10 min, centrifuged at 15,000 g for 15 min and the supernatants were removed. Twenty μ L of the internal standard solutions (2 mg/mL 1,2-didodecanoyl-*rac*-glycero-3-phosphocholine) were added to the pellet. The pellet was then extracted three times with 1 mL of a 1:1 chloroform/methanol mixture (Dreyfus et al. 1997) and evaporated to dryness. The lipid residue was suspended in 1 mL of isopropyl alcohol, filtered (0.45 μ m) at 15,000 g for 10 minutes. The filtrate was used for LC-MS analysis. Liquid chromatography was performed on a gradient HP1100 HPLC (Agilent Technologies, Santa Clara, CA) system using an aqueous mobile phase A (1% formic acid, 5 mM ammonium formate in water) and an organic mobile phase B (isopropyl alcohol, 1%

formic acid). The mobile phase flow was 0.3 mL/min, which began at a composition of 50% A and B, and was linearly ramped to 80% B over the next 30 min. We used a Phenomenex (Torrance, CA) Phenosphere 5 cm × 4.6 mm, 5 µm particle size column. The mass spectrometer was a Thermo Finnigan triple quadrupole TSQ 7000, operated in positive ion parent scan mode. The sample was introduced through an electrospray ionization source. The second quadrupole was used as a collision chamber with argon as a collision gas. Sphingomyelins, phosphatidylcholines and lyso-phosphatidylcholines were analyzed in the parent ion scan mode by monitoring all parent ions that lost m/z of 184.1, corresponding to the head group of sphingomyelins, phosphatidylcholines and lyso-phosphatidylcholines. Individual lipids were measured by integrating the area counts of the m+1 parent ions and normalizing the response to the appropriate internal standard.

Western blotting

Control and δ -ALA treated cultures were lysed in SDS sample buffer supplemented with protease and phosphatase inhibitors and protein concentrations were determined using the BCATM assay (Pierce Chemicals, Rockford, IL). Samples were analyzed on 7.5, 10.0 or 12.5%, or 4–20% gradient (Lonza, Rockland, ME) poly-acrylamide gels under reducing conditions (except for determination of MAG) and transferred to nitrocellulose or polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA). Membranes were blocked in 5% milk in Tris buffered saline with 0.05% Tween 20 (Sigma, St. Louis, MO) and incubated overnight in primary antibodies in the blocking buffer. After washing off primary antibodies, anti-mouse, anti-rabbit or anti-rat HRP-linked secondary antibodies were added for 2 hours. Bound antibodies were visualized using an enhanced chemiluminescence detection kit (PerkinElmer Life Sciences, Boston, MA). Protein carbonylation was determined as described (Ojano-Dirain et al. 2005). Films were digitally imaged using a GS-800 densitometer (Bio-Rad, Hercules, CA) and were formatted for printing using Adobe Photoshop 5.5.

Cell death assay

The effect of δ -ALA on cell death was measured with lactate dehydrogenase (LDH) toxicology assay kits (Sigma-Aldrich, St. Louis, MO). SCs and DRG neurons were treated with δ -ALA for three days in medium containing heat-inactivated serum. The medium was withdrawn at the end of treatment and assayed for LDH release. Total LDH in cells was also measured, according to the procedure described by the manufacturer. All assays were normalized to total LDH. Absorbance values for all experiments were obtained using a Benchmark microplate reader (Bio-Rad, Hercules, CA). Lactate dehydrogenase tests were performed at least three times, with six samples used at each δ -ALA concentration.

Quantification and statistical analyses

Where quantification is presented, experiments were repeated at least three independent times. Final results are presented as average $\pm$ SEM. P values were calculated by t-test, two-tailed, two samples, and unequal variance for data with two variables using Microsoft Excel. Multiple comparisons were performed by one-way ANOVA followed by Bonferroni's procedure and using GraphPad Prism 5 software.

Results

We treated cocultures of SCs and DRG neurons with δ -ALA (0.1–1.0 mM) simultaneously with the induction of myelination to determine the effects of δ -ALA on the expression of myelin-related proteins (Fig. 1). Vehicle-treated control samples synthesized abundant myelin after 10 days, as detected with antibodies to the myelin proteins MAG, P0 and PMP22 (Fig. 1A). In contrast, lysates of cocultures that were treated with δ -ALA contained

markedly reduced levels of myelin proteins, suggesting that δ -ALA interferes with myelin formation. This inhibitory effect is present at the lowest concentration of δ -ALA employed (0.1 mM) and is maximally expressed at δ -ALA concentrations of 0.3 mM or higher. The decrease in the level of MAG suggests that δ -ALA interferes with the initial stages of SC membrane expansion. To corroborate these findings, we grew cultures on glass coverslips and processed them for immunolabeling with MAG, P0 and myelin basic protein (MBP) antibodies (Fig. 1B). Specificity of the myelin-like immunoreactivity was confirmed by the absence of signal in non-specific IgG-incubated samples (Fig. 1B, inset, upper center). Control cultures contain numerous myelinated segments that were identified with the anti-myelin protein antibodies. In contrast, myelin internodes were rarely seen in samples treated with δ -ALA (0.3 mM) for 10 days (Fig. 1B). Thus, the results of biochemical and morphological studies indicate that δ -ALA inhibits myelin synthesis by peripheral glial cells.

Next, we determined the influence of δ -ALA in cocultures in which myelin was already synthesized (Fig. 2A). SC and DRG neuron cocultures myelinated for 12 days before exposure to 0.5 mM δ -ALA for 12 days. Control samples were collected prior to the initiation of δ -ALA treatment (0) and then again at the end (0*). Immunoblotting of whole culture lysates for P0 and PMP22 revealed that both control samples contained similar amounts of these myelin proteins (Fig. 2A). However, the steady-state levels of P0 and PMP22 were markedly reduced after 12 days of exposure to 0.5 mM δ -ALA. Thus, exposure of peripheral neural cells to δ -ALA leads to destruction of pre-formed myelin.

One mechanism by which δ -ALA may cause both demyelination and dysmyelination is by inducing oxidative damage to neural proteins. Therefore, we assayed the levels of carbonylated proteins in control and δ -ALA treated cultures (Fig. 2B–E). In the demyelination paradigm (Fig. 2B, C), protein carbonylation increased with δ -ALA treatment, as compared to controls. The elevated levels of protein carbonyls in the myelinated control sample (0*) may be reflective of the additional 12 day culture time. Quantification, after correction for protein loading, revealed a significant (* p <0.05) increase in protein carbonyls in δ -ALA treated samples (Fig. 2C). In the dysmyelination experiment, protein carbonylation was pronounced at 0.5 mM δ -ALA (Fig. 2D), an effect that is statistically significant (Fig. 2E). These results are consistent with the notion that myelin is prone to oxidative damage (Mronga et al. 2004; Stahnke et al. 2007) and that oxidative stress contributes to the demyelination and dysmyelination induced by δ -ALA.

Control and treated cocultures were processed for analysis of lipid content by mass-spectrometry to further examine the influence of δ -ALA exposure on peripheral neural cells (Fig. 3). The lipids chosen are representative of three classes found in PNS myelin with parent ions that lose the same fragment with m/z 184.1 and can therefore be analyzed in one chromatographic run. We measured sphingomyelin (Fig. 3A), which is more abundant in peripheral than central nervous system tissue. Phosphatidylcholines (Fig. 3B) and their derivative lyso-phosphatidylcholines (Fig. 3C) are readily measurable by the same procedure as sphingomyelin and are also enriched in myelin. We specifically chose to analyze lipids with fatty acid residues predominant in peripheral nerves. Individual lipids of three different classes were determined and plotted *versus* δ -ALA concentration (Fig. 3A–C). The levels of all lipids decreased markedly in response to increasing δ -ALA concentrations after 12 days of myelination.

To substantiate the pro-oxidant effects of δ -ALA on myelinated peripheral samples, we incubated freshly-excised teased sciatic nerves for 4 hours with 5 mM δ -ALA. Protein oxidation (carbonylation) and lipid peroxidation (hydroxynonenal and malondialdehyde) were measured by Western immunoblotting (Fig. 4). In agreement with our results in

cultured cells, we found striking increases in these indices of oxidative damage across a wide variety of proteins, particularly above 50 kDa. These results indicate that the oxidative stress induced by δ -ALA is manifested broadly among cellular proteins.

The reduction in myelin protein and lipid levels (Figs. 1–3) and the increase in markers of oxidative damage in myelinated samples (Figs. 2B–E and 4) suggest that SCs are the primary target of δ -ALA. Myelination is a highly energy-dependent process (Morell 1984; deVellis and Carpenter 2006) that makes glial cells susceptible to oxidative damage. Therefore, we asked whether δ -ALA might alter the expression of proteins involved in various aspects of mitochondrial function in isolated SCs. Lysates of control SCs and of cells exposed to δ -ALA for 72 hours were probed with a panel of antibodies against specific mitochondrial proteins, including subunits of the respiratory chain, heat shock protein 60 (HSP60) and manganese superoxide dismutase (MnSOD) (Fig. 5A). Delta-ALA did not alter the expression of HSP60, MnSOD or subunits of complexes III–V (Core protein 2 of Complex III, subunit I of Complex IV and subunit a of Complex V, respectively). In contrast, the steady-state levels of NDUF8 (Complex I), 39 kDa (Complex I) and iron protein (Ip, Complex II) were reduced by the compound in a dose-dependent manner. Quantification of independent experiments for the 39 kDa Complex I protein, Ip30 and NDUF8 revealed a statistically significant reduction in expression, when compared to controls (Bonferroni, $p < 0.001$). The differences in protein levels between the various concentrations of δ -ALA were significant for Ip30 and NDUF8 but not for the 39 kDa Complex I protein. The sustained expression of HSP60, a mitochondrial chaperone, suggests that the overall cellular mitochondrial content was largely unaffected by δ -ALA. While the levels of chaperones, such as HSP60, or antioxidant enzymes like MnSOD may increase in response to oxidative stress (Mitsumoto et al. 2002), in this paradigm they remained unchanged. However, protein activity levels, which we did not measure, may have varied even under constant protein expression (Hollander et al. 2000; Capel et al. 2004; Dowling et al. 2006; Pipinos et al. 2008).

Next, we incubated cells with the JC-1 mitotracker dye and processed them for flow cytometry to gain additional insight into the effects of δ -ALA on mitochondrial integrity (Fig. 5B). Healthy cells fluoresce orange, while cells in which mitochondria become depolarized fluoresce green (Reers et al. 1991), which may occur as a result of oxidative stress (Mronga et al. 2004). As expected, control SCs clustered in the orange range, while a shift toward green fluorescence occurred after 3 hours of exposure to 5 mM δ -ALA (Fig. 5B). Quantification of independent experiments indicated that over 50% of the δ -ALA treated cells contained mitochondria with altered polarity (Fig. 5C). Similar experiments with DRG neurons were not feasible because of the technical difficulty of flow cytometric analysis of cells that have an extended neurite network. However, unlike the morphological change induced by DCA in SCs (Felitsyn et al. 2007), exposure to δ -ALA did not alter the general appearance of SCs or neurons when observed by phase microscopy (data not shown).

To further investigate the apparent lack of δ -ALA induced damage on neuronal proteins, we probed whole cell lysates of purified DRG neuron cultures with a panel of antibodies against neurofilament and mitochondrial proteins (Fig. 6). As shown in the Western blot, we did not detect noticeable changes in the expression of neurofilament heavy, medium or light subunits even after exposure to 1.0 mM δ -ALA for six days (Fig. 6A). Immunostaining of δ -ALA-treated cultures with the anti-neurofilament antibodies revealed the presence of a dense neurite network that was similar to control samples (data not shown). Thus, results from both biochemical and morphological analyses indicate that inhibition of myelination by δ -ALA is not mediated by direct damage to neurites. Consistent with this interpretation, we found that the steady-state expression of several mitochondrial proteins of isolated DRG

neurons were unaffected by a six day δ -ALA treatment (Fig. 6B). While there is a slight reduction in the expression of Ip30 in δ -ALA treated neuron cultures, semi-quantitative analysis of independent experiments did not show a statistically significant effect ($p=0.1168$). These results are in contrast to the reduced expression of mitochondrial proteins in SCs after three days of δ -ALA exposure (Fig. 5A), and this indicates that SCs are more vulnerable to δ -ALA-mediated disruption of mitochondrial oxidative phosphorylation, than DRG neurons.

As an additional quantitative measure of δ -ALA toxicity, we assayed LDH release into the medium as an indicator of cell death (Fig. 7). Exposure to δ -ALA was associated with a small, but statistically significant and dose-dependent increase in cell death in non-myelinating SCs (Fig. 7A). Nonetheless, the actual percentage of dying cells was very small (5.8% in control vs. 7.5%) even at the highest tested concentration of 0.5 mM δ -ALA (Fig. 7A). In comparison, a six day δ -ALA exposure (0.1–0.5 mM) did not alter LDH release significantly from DRG neurons (Fig. 7B). Together, these results are consistent with the hypothesis that SCs are more susceptible than peripheral neurons to δ -ALA toxicity.

Discussion

These data indicate that δ -ALA exposure causes striking changes in myelin formation and maintenance in peripheral nerve cells. The effect is dose-dependent and appears to be mediated primarily by altering glial cell biology. The process of myelination and myelin maintenance depends on various adhesive and signaling events between glial cells and axons and is highly sensitive to environmental and genetic perturbations (Nave and Salzer 2006; Trajkovic et al. 2006). Although demyelination can be a secondary consequence of axonal damage, SCs appear to be more susceptible than DRG neurons to the toxic effects of δ -ALA. The heightened vulnerability of myelinating glia to oxidative damage, as compared to other neural cell types, is in agreement with studies reporting white matter damage in paradigms of oxidative stress (Juurlink et al. 1998; Bartzokis 2004; Beal 2004).

SCs are known to express constituents of the porphyrin-heme biosynthetic pathway and incorporate δ -ALA as a precursor for the synthesis of porphyrin intermediates (Whetsell et al. 1984). Porphyrin-like fluorescence was detected within myelinated SCs when organotypic mouse DRG cultures were exposed to 10 mM δ -ALA for 48 h (Whetsell et al. 1984). In these experiments, δ -ALA was used as a marker of myelin rather than a test compound of interest. Concentrations of δ -ALA used in *in vitro* studies have ranged from 0.05–50 mM (Whetsell et al. 1984; Hermes-Lima et al., 1992; Princ et al. 1997; Carneiro and Reiter 1998; Princ et al. 1998; Juknat et al. 2003; Onuki et al. 2004; Adhikari et al. 2006), in contrast to levels of 1–8 μ M measured in human blood or plasma (Sakai and Morita 1996; Floderus et al. 2006). We used concentrations of δ -ALA lower than those encountered in the vast majority of *in vitro* studies, and we exposed the cultures for extended periods (Figs. 1–3). Such a low-dose treatment paradigm may mimic the *in vivo* conditions encountered in subjects chronically exposed to chemicals, such as DCA or lead, or to diseases, such as hereditary porphyria (Becker and Kramer 1977; Gorchein and Webber 1987) or tyrosinemia type I (Mitchell et al. 1990) that cause δ -ALA to accumulate. Additional experimental data support the concept that myelinating glial cells are targets of δ -ALA. For example, δ -ALA has an affinity for MBP, a cytosolic structural component of compact myelin. Intracranial injection of ^{14}C - δ -ALA in experimental animals detected almost half of the isotope bound to basic proteins (Muzyka and Moks 1984; Muzyka and Bogovskii 1985), despite the greater affinity of δ -ALA for Wolfram proteins. Since MBP is a common constituent of both central and peripheral myelin, it could be expected that ^{14}C - δ -ALA also labeled peripheral myelin.

Our studies reveal a differential effect of δ -ALA on several protein subunits of the respiratory chain in SCs (Fig. 5). The diminished expression of specific subunits in complexes I and II may not reflect the full extent of δ -ALA's adverse influence on the integrity of the respiratory chain components. Although the functional impact of these changes on oxidative phosphorylation was not addressed in this study, it is reasonable to speculate that mitochondrial energy failure may have contributed to the disruption of myelin formation, a process that requires ATP (Morell 1984; deVellis and Carpenter 2006). Less is known about the energy requirements of mature myelin; however, it is likely to be sensitive to metabolic insults because of its highly specialized membrane domains (Morell and Toews 1996). The decrease in SC viability following exposure to δ -ALA (Fig. 7A) could in the long-term also contribute to the myelin defects and demyelination we observed.

A possible mechanism accounting for δ -ALA's toxicity is oxidative damage to SCs, as manifested by an increase in protein carbonylation and mitochondrial membrane depolarization (Figs. 2, 4, 5). Delta-ALA is known to have pro-oxidant properties (Demasi et al. 1996; Princ et al. 1997; Princ et al. 1998; Juknat et al. 2003; Onuki et al. 2004; Adhikari et al. 2006; Bechara et al. 2007), and we observed protein carbonylation in myelinating SCs (Fig. 2). Exposure of excised peripheral nerves to δ -ALA identified a pronounced effect of the compound on protein carbonyls and lipids that was exerted presumably in part by oxidative damage to myelin. Thus, oxidative stress induced in cells by δ -ALA may arise from two potential mechanisms. One mechanism may be mediated by direct damage to cellular proteins caused by the pro-oxidant structural feature of δ -ALA, in which the loss of an electron from the keto group oxygen would generate a free radical. Another potential pathway involves increased production of reactive oxygen species from an impaired electron transport chain. This could initiate a vicious cycle of progressive accumulation of mitochondria-derived free radicals coupled with progressive mitochondrial energy failure. Both of these events would contribute to disruption of myelin metabolism and cell dysfunction.

Elevated levels of δ -ALA are observed in hereditary tyrosinemia and porphyrias. Patients display neurological symptoms involving the PNS in both of these disorders. Nerve conduction velocity studies in such patients suggest that axonal degeneration is the primary effect of δ -ALA exposure, followed by demyelination (Flugel and Druschky 1977; Mitchell et al. 1990). However, on histological examination, both demyelination and degeneration of peripheral nerves were observed (Gibson and Goldberg 1956; Becker and Kramer 1977; Flugel and Druschky 1977). The former group of patients (Gibson and Goldberg 1956), were administered barbiturates, which are known to precipitate large increases of δ -ALA levels (Zimmer et al. 1980). Coincidentally, these patients almost uniformly developed neurological symptoms, including those consistent with peripheral neuropathy, which may have been due to an increase in δ -ALA. Our findings support the deleterious effect of δ -ALA on peripheral nerve cells, particularly Schwann cells. However, accumulation of other potential neurotoxins occurs in porphyria (Becker and Kramer 1977), tyrosinemia (Russo et al. 2001) and DCA exposure (Stacpoole et al. 2006; Shroads et al. 2008). Thus, δ -ALA may not be the only factor contributing to peripheral neuropathy in these conditions.

Delta-ALA is also elevated during lead poisoning due to disturbances in heme metabolism (Sakai et al. 2000). Lead is known to substitute for zinc in cysteine-rich, metal-binding sites of proteins, including δ -ALA dehydratase (Silbergeld 1992), which in turn leads to accumulation of δ -ALA. Lead exposure in humans produces striking effects on peripheral nerve function, resulting in loss of coordination and weakness in the extremities (Audesirk 1985). Chronic lead intoxication in adult rodents is associated with demyelination of peripheral axons due to direct injury to SCs (Powell et al. 1982; Coria et al. 1984). Our results suggest that the mediator of this SC damage is δ -ALA.

In a previous report, we described the adverse effect of DCA on myelination in SC-DRG neuron cocultures (Felitsyn et al. 2007). A possible mechanism that would reconcile data from our previous report (Felitsyn et al. 2007) and the current findings invokes an initial inhibitory action by DCA on tyrosine catabolism in SCs, followed by accumulation of δ -ALA. Although the effects we observed with DCA were at concentrations more than one order of magnitude higher than those used here for δ -ALA, this could be due to a lower intrinsic potency of DCA acting directly on cells, and/or to the requirement for such high DCA doses in tissue culture to inhibit tyrosine metabolism. Neither DCA nor δ -ALA exposure adversely changed the levels of neurofilament proteins, so neuronal toxicity is unlikely to account for the effects of these compounds on myelin metabolism.

In summary, δ -ALA exposure caused dys- and demyelination in cocultures of SCs and peripheral neurons. Both the pro-oxidant property of δ -ALA itself and leakage of oxygen-free radicals from a damaged respiratory chain may contribute to a state of heightened oxidative stress predominantly in glial cells. This in turn could lead to peripheral neurotoxicity under hereditary or acquired conditions in which the accumulation of δ -ALA occurs.

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Abbreviations used

δ-ALA	delta-aminolevulinate
DCA	dichloroacetate
DMEM	Dulbecco's Modified Eagle Medium
DRG	dorsal root ganglion
FCS	fetal calf serum
GSH	glutathione
HRP	horseradish peroxidase
Ip	iron protein
LDH	lactic dehydrogenase
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MEM	minimum essential medium
NF	neurofilament
PMP22	peripheral myelin protein 22
P0	myelin protein zero
SC	Schwann cell

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Figure 1. Exposure of neuron and glia cocultures to δ -ALA blocks myelin formation

(A) Myelinating SC-DRG neuron cultures were treated with δ -ALA for 10 days under myelination-promoting conditions. Whole cell lysates (10 μ g/lane for MAG and P0; 20 μ g/lane for PMP22) were analyzed by Western blotting. A non-specific protein reactive with the anti-PMP22 antiserum is indicated by the arrowhead. Alpha-tubulin was used as the loading control. Molecular mass in kDa. (B) Representative cultures were immunolabeled with monoclonal antibodies against MAG, P0 and MBP. A sample incubated with non-specific mouse IgG is shown (inset in upper right panel). Scale bar, 20 μ m.

Figure 2. Enhanced protein carbonylation is associated with demyelination and dysmyelination induced by δ-ALA

(A) Demyelination induced by δ-ALA exposure. Cocultures underwent myelination for 12 days at which point the first control (0 mM δ-ALA) was obtained and δ-ALA treatment was initiated. This control was used to document myelin formed at this stage. After 12 days, the second control (0* mM δ-ALA) and cells exposed to 0.5 mM δ-ALA were analyzed (20 μg/lane) together for the myelin proteins PMP22 and P0. (B) Protein carbonylation during demyelination. Same conditions were used as in (A). For details of determining protein carbonylation see Methods. (C) Quantification of data in (B) using one-way ANOVA with post-hoc Bonferroni test, *p < 0.05. (D) Protein carbonylation during dysmyelination. Exposure to δ-ALA occurred during the first 12 days of myelination, after which the samples were lysed and analyzed (20 μg protein/lane) for protein carbonyls. Alpha-tubulin (A, B) or GAPDH (D) are shown as protein loading controls. Molecular mass in kDa. (E) Quantification of data in (D) using one-way ANOVA with post-hoc Bonferroni test, *p < 0.05.

Figure 3. Sphingomyelins (A), phosphatidylcholines (B), and lyso-phosphatidylcholines (C) decrease in myelinating SC-DRG neuron cocultures after δ -ALA exposure

Lipids were determined by liquid chromatography-mass spectrometry as described in the Methods. (A) Sphingomyelins. The differences between control and δ -ALA (0.25 and 0.50 mM) treated cultures were significant for normalized intensities of the parent ions with m/z 759.8, 785.9, 787.9 and 813.9 ($p < 0.01$, Bonferroni). The normalized intensity for parent ion with m/z 785.9 was scaled up 10 times to visualize it better. (B) Phosphatidylcholines. The differences of normalized intensity between control and 0.25 mM δ -ALA were significant for all parent ions ($*p < 0.001$, Bonferroni), excluding m/z 806.7 (non-significant). The differences between control and 0.50 mM δ -ALA were also significant for all parent ions

(* $p < 0.001$, Bonferroni), excluding m/z 806.7. The normalized intensity for parent ion with 760.7 m/z was scaled down 10 times to improve data presentation. (C) Lyso-phosphatidylcholines. The differences of normalized intensities between control and 0.25 mM δ -ALA and control and 0.50 mM δ -ALA were all significant (* $p < 0.001$, Bonferroni).

Figure 4. *Ex vivo* treatment of sciatic nerves with δ -ALA causes increased protein carbonylation and lipid peroxidation

Freshly-excised sciatic nerves were placed in MEM. One of the nerves from each pair was exposed to 5 mM δ -ALA and incubated together with control nerves at 37 °C and 5% CO₂ for 4 hours. Subsequently, nerves were lysed and analyzed (20 μ g/lane) for protein carbonylation and lipid peroxidation (hydroxynonenal and malon-dialdehyde). GAPDH was used as the protein loading control. =C=O refers to protein carbonylation. Molecular mass in kDa.

Figure 5. The effects of δ -ALA on mitochondrial proteins and polarity

(A) SCs were exposed to δ -ALA for 72 hours and analyzed (20 μ g/lane) for the expression of various subunits of the mitochondrial respiratory chain complexes (CI-V), HSP60 and MnSOD. The specific subunits of corresponding complexes are indicated in parentheses. Alpha-tubulin was used as the protein loading control. Molecular mass in kDa. (B) Cultured SCs were exposed to serum-free DMEM with and without δ -ALA for 3 hours. Cells were then treated with 10 μ M JC-1, washed, trypsinized and resuspended in 10% FCS in DMEM. Samples were analyzed by flow cytometry for orange (595 nm) and green (525 nm) fluorescence. (C) Quantification of the fraction of cells in the R2 area in (B), *p<0.05.

Figure 6. The expression of neuronal proteins are largely unaffected by exposure to δ -ALA
 Dissociated DRG neuron cultures were purified through three cycles of fluorodeoxyuridine treatment. After a six day wash-out period, cultures were exposed to δ -ALA for six days. (A) Whole cell lysates (5 μ g/lane) were analyzed for several isoforms of neurofilament proteins, including neurofilament subunits, heavy (NF-H), medium (NF-M) and light (NF-L). (B) Whole neuronal protein lysates (20 μ g/lane) were blotted for constituents of mitochondrial complexes I–V (CI–V). The specific subunits of corresponding complexes are indicated in parentheses. The levels of HSP 60 and MnSOD were also analyzed. Alpha-tubulin was used as the protein loading control. Molecular mass in kDa.

Figure 7. LDH release from SCs and DRG neurons in response to δ -ALA

(A) SCs were exposed to the indicated concentrations of δ -ALA for three days prior to analysis. Each data point represents the average of six independent values. All values were normalized to total LDH, * $p < 0.001$. (B) For the analysis of DRG neurons, the same procedure was followed as in (A).

Table 1

Primary antibodies used in this study

Species	Antigen	Source and Catalog #	Dilution	
			WB*	IS**
Rabbit	PMP22	Notterpek et al. 1999; n/a	1:1000	n/a
Mouse	P0	Archelos et al. 1993; n/a	1:1000	1:500
Rat	MBP	Chemicon, Temecula, CA; MAB386	1:250	1:500
Mouse	MAG	Chemicon, Temecula, CA; MAB1567	1:500 [§]	1:250
Mouse	α -Tubulin	Sigma, St. Louis, MO; T6199	1:4000	n/a
Mouse	NF-L	Dako North America, Inc., Carpinteria, CA; M0762	n/a	1:1000
Mouse	GAPDH	Encor Biotechnology Inc., Alachua, FL; MCA-1D4	1:5000	n/a
Mouse	NF-L	Encor Biotechnology Inc., Alachua, FL; MCA-DA2	1:5000	1:1000
Mouse	NF-M	Encor Biotechnology Inc., Alachua, FL; MCA-3H11	1:5000	n/a
Mouse	NF-H	Encor Biotechnology Inc., Alachua, FL; 3G3	1:500	n/a
Mouse	OxPhos	Mitosciences, Eugene, OR; MS604	1:250	n/a
Mouse	HSP60	Stressgen Biotechnologies, Victoria, Canada; SPA-806F	1:1000	n/a
Mouse	C I 39 kDa	Invitrogen Corporation, Carlsbad, CA; A21344	1:500	n/a
Rabbit	Dinitrophenyl (for protein carbonyls)	Sigma, St. Louis, MO; D9656	1:5000	n/a
Rabbit	HNE	Alpha Diagnostics Int., San Antonio, TX; HNE 11S	1:1000	n/a
Rabbit	MDA	Alpha Diagnostics Int., San Antonio, TX; MDA11S	1:500	n/a
Rabbit	MnSOD	Stressgen Biotechnologies, Victoria, Canada; SOD11-0D	1:2000	n/a

* Western Blot,

** Immunostain

[§] non-reduced gel

n/a – non-applicable