ORIGINAL ARTICLE

Nervonic Acid Attenuates Accumulation of Very Long-Chain Fatty Acids and is a Potential Therapy for Adrenoleukodystrophy

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Abstract

Adrenoleukodystrophy (ALD) is an X-linked inherited peroxisomal disorder due to mutations in the ALD protein and characterized by accumulation of very long-chain fatty acids (VLCFA), specifically hexacosanoic acid (C26:0). This can trigger other pathological processes such as mitochondrial dysfunction, oxidative stress, and inflammation, which if involves the brain tissues can result in a lethal form of the disease called childhood cerebral ALD. With the recent addition of ALD to the Recommended Uniform Screening Panel, there is an increase in the number of individuals who are identified with ALD. However, currently, there is no approved treatment for pre-symptomatic individuals that can arrest or delay symptom development. Here, we report our observations investigating nervonic acid, a monounsaturated fatty acid as a potential therapy for ALD. Using ALD patient-derived fibroblasts, we examined whether nervonic acid can reverse VLCFA accumulation similar to erucic acid, the active ingredient in Lorenzo's oil, a dietary intervention believed to alter disease course. We have shown that nervonic acid can reverse total lipid C26:0 accumulation in a concentration-dependent manner in ALD cell lines. Further, we show that nervonic acid can protect ALD fibroblasts from oxidative insults, presumably by increasing intracellular ATP production. Thus, nervonic acid can be a potential therapeutic for individuals with ALD, which can alter cellular biochemistry and improve its function.

Keywords Very long-chain fatty acids · Adrenoleukodystrophy · Dietary lipids · Monounsaturated fatty acids · Peroxisomes · Sphingomyelin · Fibroblasts

Introduction

Adrenoleukodystrophy (ALD) is an X-linked inherited peroxisomal neurodegenerative disorder characterized by mutations in the *ABCD1 (ATP binding cassette subfamily D member 1)* gene, which encodes for the ALD protein (ALDP). ALDP is a transmembrane protein responsible for the transportation of very long-chain fatty acids (VLCFA) into peroxisomes for further degradation [1, 2]. Defects in ALDP are linked to the pathogenic accumulation of saturated VLCFA, specifically hexacosanoic acid (C26:0), particularly in plasma, brain white matter, and the adrenal cortex [3]. This increase in levels of saturated VLCFA in ALD can be attributed to its enhanced biosynthesis in cells which are also defective in peroxisomal beta-oxidation, which results in further VLCFA elongation by ELOVL1 (elongation of very-long-chain fatty acids 1), an elongase enzyme [4, 5]. While the pathogenesis of ALD is incompletely understood, it involves an imbalance of biosynthesis and degradation of saturated VLCFA that results in tissue injury due to secondary pathological processes such as destabilizing multilamellar membrane structure of myelin [6]. While C26:0 is the main diagnostic marker for ALD, its abnormal accumulation triggers a cascade of downstream processes including cellular oxidative stress, mitochondrial dysfunction, and inflammation that eventually elicits apoptotic cell death [1, 2, 7].

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ALD manifests as several different conditions such as childhood cerebral ALD (cALD), adrenomyeloneuropathy (AMN), and Addison's disease. Being a progressive disease, early diagnosis of ALD is paramount. Newborn screening for ALD was initiated following its addition to the Recommended Uniform Screening Panel (RUSP) in February 2016 [8]. Since then, the number of newborns identified with ALD has increased. While there are standard protocols such as serial monitoring and genetic counseling for boys diagnosed with ALD, there are only limited treatments available that can arrest the disease. Currently, there is no cure for ALD. The available therapeutic options include hematopoietic stem cell transplantation (HSCT) and hormonal replacement therapy which are offered to patients once they become symptomatic [2, 9]. Recently, a lentiviral vector-mediated autologous hematopoietic stem cell gene therapy was shown to be a safe and effective alternative to HSCT in boys with early-stage cALD [10]. Though effective, HSCT has safety concerns and neither HSCT nor ex vivo gene therapy is offered to pre-symptomatic boys.

Lorenzo's oil (LO) was once considered a therapeutic option for ALD, especially in asymptomatic or pure AMN patients [11]. LO is a 1:4 triglyceride mixture of erucic acid (C22:1) and oleic acid (C18:1) that has been shown to significantly reduce plasma C26:0 levels and can slow down the development of cALD during childhood [2, 12]. However, it did not show a significant effect on preventing the development of demyelinating lesions in ALD patients [13]. Due to contradictory and unclear benefits, the commercial development of LO was terminated recently in the USA. Moreover, preliminary animal studies showed erucic acid to be associated with cardiotoxicity, thus making it a controversial therapeutic choice for ALD [14]. Studies with other monounsaturated fatty acids such as nervonic acid (C24:1) have been limited but have shown potential as a therapeutic option [15, 16].

We conducted a systematic, controlled study using patient-derived fibroblasts to explore the biochemical benefit of nervonic acid in ALD. We used erucic acid, the active ingredient in LO, as a comparator. Further, we also conducted functional studies to characterize additional pharmacological properties of nervonic acid that can offer cytoprotection. With the lack of any approved therapies for newly diagnosed patients with an ALD mutation, nervonic acid may be a safe and effective therapeutic option that can delay or potentially arrest disease progression.

Methods

Chemicals and Reagents

Nervonic acid and erucic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Minimum essential media (MEM), heat-inactivated fetal bovine serum (FBS), MEM non-essential amino acids solution, penicillin–streptomycin, and trypsin–EDTA (0.05%) were obtained from Life Technologies (Carlsbad, CA, USA). Phosphate-buffered saline (PBS) was acquired from Genesse Scientific (San Diego, CA, USA). Nervonic acid and erucic acid stock solutions were prepared in ethyl alcohol (EtOH, 200 Proof) from Pharmco-Aaper (Brookfield, CT, USA).

Fibroblast Culture and Treatments

Human dermal fibroblasts, AMN (GM17819) and cALD (GM04904) cell lines were purchased from Coriell Institute (Camden, NJ, USA). Human dermal fibroblasts derived from neonatal foreskins (NHDF; Lonza, Basel, CH) were used as control cell line labeled as normal. NHDF was a generous gift from Dr. James Dutton (Stem Cell Institute, University of Minnesota, Minneapolis, MN, USA). Fibroblasts were cultured in 60 mm dishes using MEM supplemented with 10% FBS, 1% MEM non-essential amino acids solution and 1% penicillin–streptomycin in a humidified incubator at 37 °C with 5% CO_2 [17]. At 70–80% confluence, cells were treated with nervonic acid (5, 20, and 50 μM), erucic acid (5, 20, and 50 μM), EtOH (vehicle), or PBS using 2% FBS media with supplements. Five days after the treatment, cells were washed with PBS and harvested using trypsin–EDTA. Cells were collected in microtubes (1.7 mL) and centrifuged at $3000 \times g$ for 10 min followed by a wash with PBS. Fibroblasts were frozen at – 80 °C until the lipid analysis.

In another set of assays, fibroblasts were seeded in 96-well plates at a density of 3×10^4 cells/well using 2% FBS media and kept overnight. The following day, cells were incubated with increasing concentrations of nervonic acid, erucic acid, or EtOH, or PBS for 5 days, following which the cell viability and ATP production were assessed. In all experiments, cells were treated with fresh nervonic acid and erucic acid solution. The maximal concentration of EtOH in the media was 0.1% or lower for fatty acids and vehicle groups.

Analysis of VLCFA and Complex Lipids

The total fatty acids were extracted from cell lysates. The protein content and the total saturated fatty acids in the extract were measured using gas chromatography-mass spectrometry (GC/MS) [18]. Total saturated VLCFA is the sum of C23:0, C24:0, C25:0, C26:0, C28:0, and C30:0. The complex lipids were measured using liquid chromatography-mass spectrometry (LC/MS) [19–21].

Cell Viability and ATP Production Assay

The viability of cells was evaluated using a DNA-based assay with CyQUANT™ NF cell proliferation assay kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Fluorescence was measured at 485 nm/530 nm wavelength using a Synergy 2 microplate reader (BioTek, Winooski, VT, USA). Cell survival results are presented as a percentage of control cells.

ATP production in cells was assayed using the ATPlite™-luminescence ATP detection assay system (Perkin-Elmer, Boston, MA, USA) according to the manufacturer's protocol. The luminescence was quantified using a Synergy 2 microplate reader (BioTek, Winooski, VT, USA) and the ATP values were represented as a percentage of the control.

Statistical Analysis

All results were presented as the mean±standard error of the mean (SEM). Lipid analyses are based on data from two independent experiments performed in duplicates, while cell function analyses are based on data from at least three independent experiments performed in triplicates. Data were analyzed using one-way analysis of variance (ANOVA) with a Dunnett's correction for multiple comparisons, or by using the unpaired Student's t test. A p value < 0.05 was considered statistically significant. All statistical analysis was performed using Graph-Pad Prism 8 software (La Jolla, CA, USA). R version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria) was used for data manipulation and visualization where appropriate [22].

Correlation analysis was performed for each of the pairs of fatty acid: C26:0 vs. C22:1; C26:0 vs C24:1; and C26:0 vs. C18:1, where a correlation coefficient (r) also known as Pearson product-moment correlation coefficient or Pearson correlation coefficient was reported to represent the degree of linear association between the pairs of variables. Categorization for interpretation for r is arbitrary, and the strength of the correlation is the same irrespective of the directionality. In this study for absolute values of r, we considered values between 0 and 0.19 as very weak, 0.2 and 0.39 as weak, 0.40 and 0.59 as moderate, 0.6 and 0.79 as strong, and 0.8 and 1 as very strong correlation.

Results

Nervonic Acid Significantly Decreases the Intracellular Accumulation of Total Lipid C26:0 and Total Saturated VLCFA

Incubation with increasing concentrations of erucic or nervonic acid showed both monounsaturated fatty acids to decrease levels of C26:0 and total saturated VLCFA. However, nervonic acid showed a consistent concentration-dependent decrease in VLCFA, especially in the AMN cell line (Fig. 1). The extent of reduction of C26:0 level was dependent on the cell lines and fatty acid. In general, compared to erucic acid, a higher concentration of nervonic acid was required to achieve a significant reduction of C26:0 and total saturated VLCFA. Notably, in normal fibroblasts, both fatty acids significantly decreased only the levels of total saturated VLCFA, but not C26:0 (Fig. 1). However, among the two fatty acids, nervonic acid did not have a further decrease in normal C26:0 levels while erucic acid caused a slight decrease in C26:0 in normal cells at increasing concentrations.

Higher Concentrations of Nervonic Acid Can Significantly Decrease the Accumulation of C26:0 in Complex Lipids

In addition to total lipid VLCFA, we also examined the status of several complex lipids. In both ALD cell lines, nervonic acid steadily decreased the levels of C26:0-ceramides and C26:0-sphingomyelin in a concentration-dependent manner (Fig. 2b, d). However, a high concentration of nervonic acid (50 μM) was required to lower the levels of C26:0-LPC (lyso-phosphatidylcholines) (Fig. 2f). In contrast, erucic acid drastically reduced the levels of these complex lipids in both ALD cells, and this effect did not follow a concentration-dependent response (Fig. 2a, c and e). In normal fibroblasts, both nervonic acid and erucic acid further reduced all complex lipids analyzed (Fig. 2a–f), and the consequence of which is unclear.

Monounsaturated Fatty Acid Treatment Alters Other Cellular Lipids

Since nervonic acid is an important component of the myelin sheath, we examined how fatty acid supplementation altered C24:1-in total lipid fatty acid and complex lipid profiles in these cells. As expected, both erucic acid and nervonic acid increased C24:1 level in the cells occurring both as total lipid VLCFA and with complex lipids (Fig. 3). The addition of increasing concentrations of nervonic acid increased the total lipid C24:1 levels by over 3-folds in comparison to erucic acid treatment. However, the levels of C24:1-ceramides and C24:1-sphingomyelins were comparable between both fatty acids in all three cell lines tested except in cALD cells at 50 μM erucic acid (Fig. 3a–f).

Similarly, we also examined the changes in levels of C24:0 in complex lipids. While we observed significant reductions in C24:0-ceramides in ALD cell lines with both fatty acids, nervonic acid showed a concentration-dependent response on all 3 cell lines tested. However, there was no
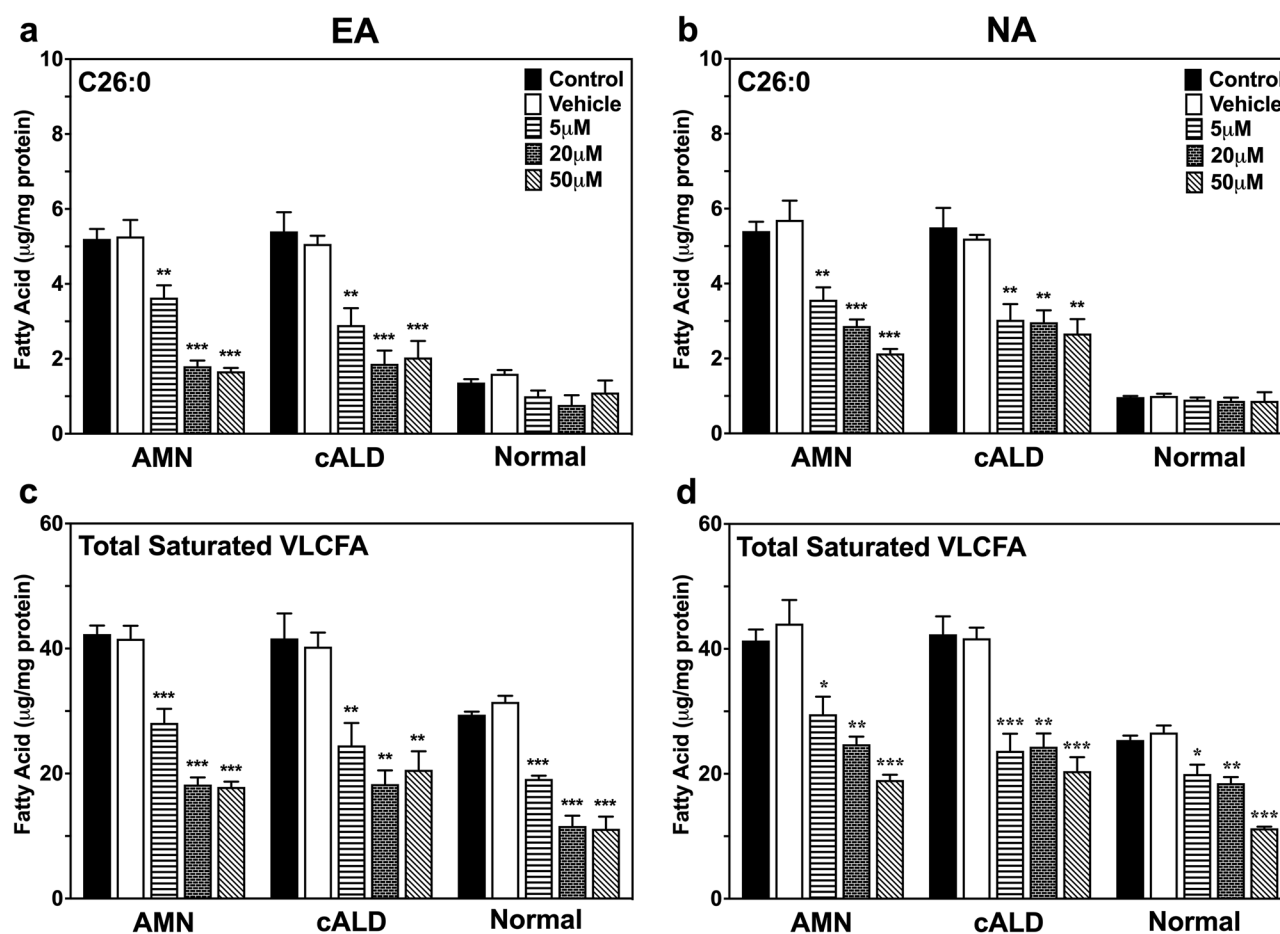
Fig. 1 Nervonic acid (NA) decreases C26:0 and total saturated VLCFA similar to erucic acid (EA). C26:0 and total saturated VLCFA levels in AMN (GM17819), cALD (GM04904) and normal (NHDF) fibroblasts treated with increasing concentrations of EA (**a**, **c**) or NA (**b**, **d**) for 5 days. The concentrations were determined using GC/MS. Data are normalized to total cellular proteins and expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistical significance from vehicle-treated cells using one-way ANOVA and Dunnett's post hoc test

significant effect on LPCs with both erucic acid and nervonic acid (Supplementary Fig. 1). We also observed no changes in C24:0-sphingomyelin levels (data not shown).

The addition of monounsaturated fatty acids can lead to its chain elongation resulting in the production of C26:1. To assess this, we measured levels of C26:1 total lipid fatty acids and C26:1-LPC. We observed a significant elevation in C26:1 level with increasing concentrations of erucic acid and nervonic acid (Fig. 4a, b). A higher concentration of nervonic acid (50 μM) showed double the amount of C26:1 fatty acid compared to the highest elevation observed with erucic acid, which was at lower concentrations in both ALD cell lines. This effect was even more with C26:1-LPC, even at lower fatty acid concentrations, especially in cALD cells (Fig. 4c, d). In normal fibroblast, only total lipid C26:1 fatty acid showed an increase with no effect on the levels of C26:1-LPC.

Nervonic Acid Offers Cellular Protection of ALD Fibroblasts from Oxidative Insults

To assess the functional benefits of fatty acid treatment on fibroblasts, especially regarding its susceptibility to oxidative cell death, cells pretreated with fatty acid were stressed using 200 μM H_2O_2. This H_2O_2 concentration was selected based on its observed ability to cause ~ 50–60% cell death in all cell lines tested. The H_2O_2-induced oxidative stress decreased the cell viability in AMN and cALD cells up to $45.3 \pm 0.6\%$ and $71.2 \pm 4.1\%$, respectively (Fig. 5a, b). These results indicate that cALD cells are more vulnerable to oxidative stress and death than the AMN cells. This may be related to a greater imbalance of enzymatic and non-enzymatic antioxidant defense in cALD cells. Upon treatment with fatty acids, nervonic acid at 5 and 20 μM protected the
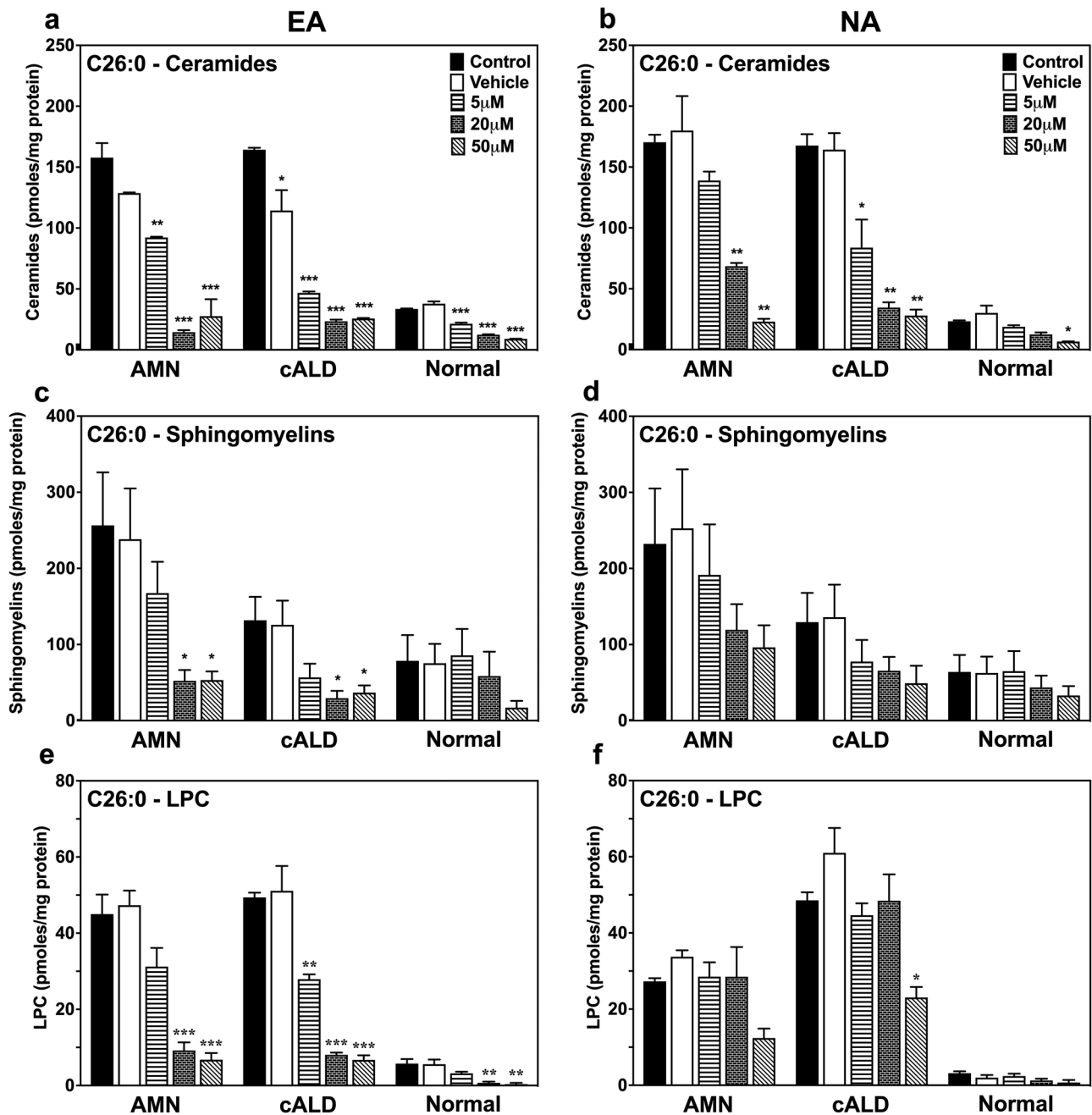
Fig. 2 Effect of nervonic acid (NA) on complex lipid profiles. C26:0-LPC (lyso-phosphatidylcholines), C26:0-ceramides, and C26:0-sphingomyelins levels in AMN (GM17819), cALD (GM04904), and normal (NHDF) cells treated with increasing concentrations of erucic acid (EA, **a**, **c**, and **e**) or NA (**b**, **d**, **f**) for 5 days. The measurements were conducted using LC/MS. Data are normalized to total cellular proteins and expressed as mean $\pm$ SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ indicate statistical significance from vehicle-treated cells using one-way ANOVA and Dunnett's post hoc test

ALD cells and significantly improved survival against the H_2O_2-induced oxidative stress. Cytoprotection was also observed at lower concentrations of erucic acid; however, nervonic acid exerted robust protection, especially in cALD cells. In normal fibroblasts, H_2O_2 reduced cell viability by $37.2 \pm 7.1\%$, with erucic acid significantly improving viability following H_2O_2-induced oxidative stress (Fig. 5c).
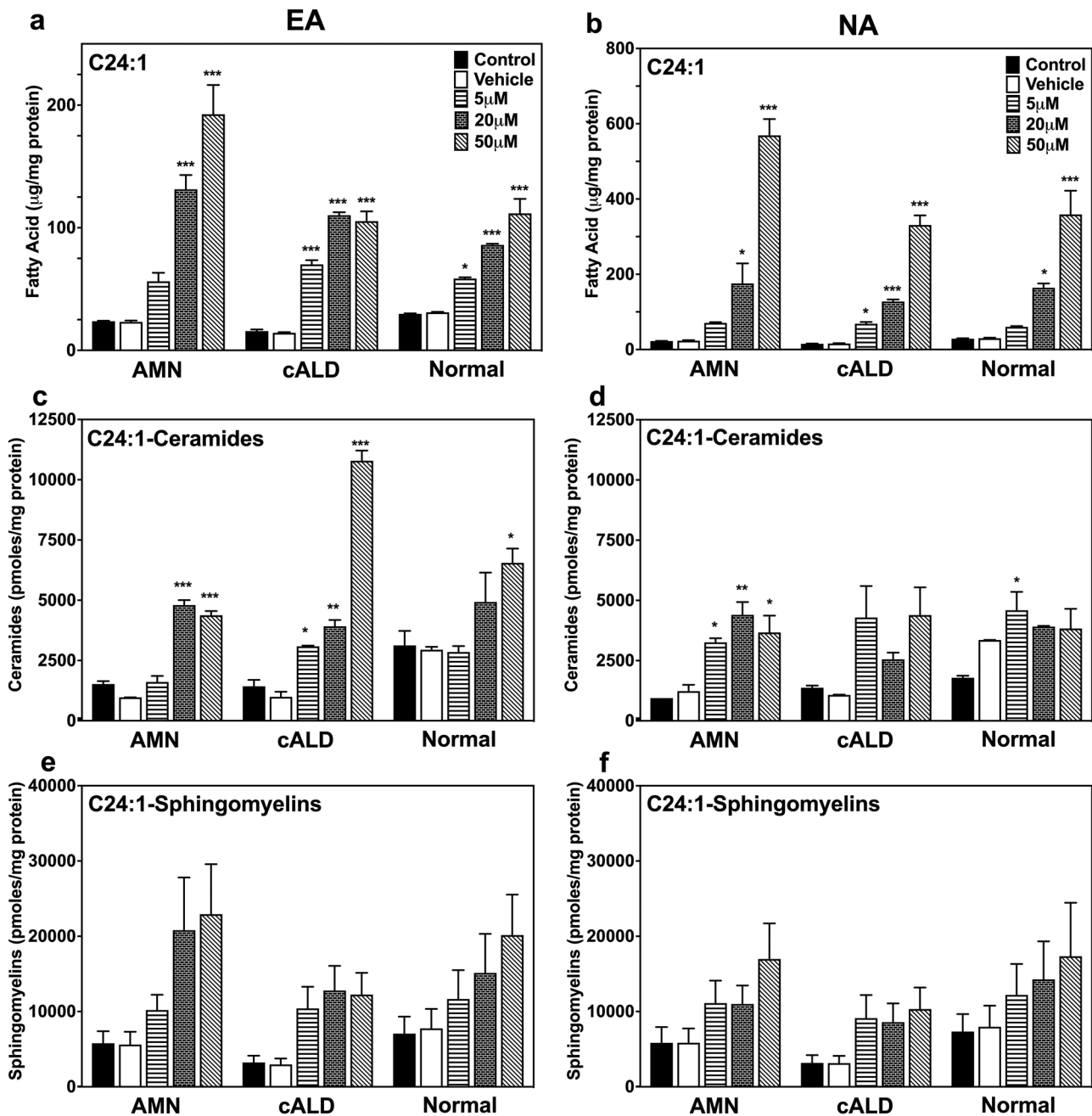
Fig. 3 Effect of nervonic acid (NA) on C24:1 total lipid fatty acids and C24:1 levels in complex lipids. C24:1 levels in AMN (GM17819), cALD (GM04904), and normal (NHDF) fibroblasts treated with erucic acid (EA, **a**, **c**) or NA (**b**, **d**) for 5 days. The total lipid fatty acid measurements were conducted by using GC/MS. The complex lipids-sphingomyelins and ceramides were assayed by LC/MS/MS. Data are normalized to total cellular proteins and expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistical significance from vehicle-treated cells using one-way ANOVA and Dunnett's post hoc test

Nervonic Acid, but not Erucic Acid Increases Intracellular ATP Production in ALD Fibroblasts

We also evaluated the potential of both fatty acids to alter the basal energy production measured as ATP content in ALD cells. We observed that following a 5-day treatment with nervonic acid, cellular ATP content was increased in cALD cells (Fig. 6). This effect was significant compared to vehicle-treated cells at 20 μM nervonic acid, while a trend was observed at other concentrations in cALD and AMN cells. A similar concentration of erucic acid (20 μM) did not show a marked increase in energy production indicating nervonic acid to be functionally beneficial in addition to its direct biochemical effects.

Fig. 4 Effect of nervonic acid (NA) on C26:1-VLCFA. C26:1 total lipid fatty acid and C26:1-LPC level in AMN (GM17819), cALD (GM04904), and normal (NHDF) fibroblasts treated with erucic acid (EA, **a**, **c**) or NA (**b**, **d**) for 5 days. Lysates were analyzed using GC/MS. Data are normalized to total cellular proteins and expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistical significance from vehicle-treated cells using one-way ANOVA and Dunnett's post hoc test
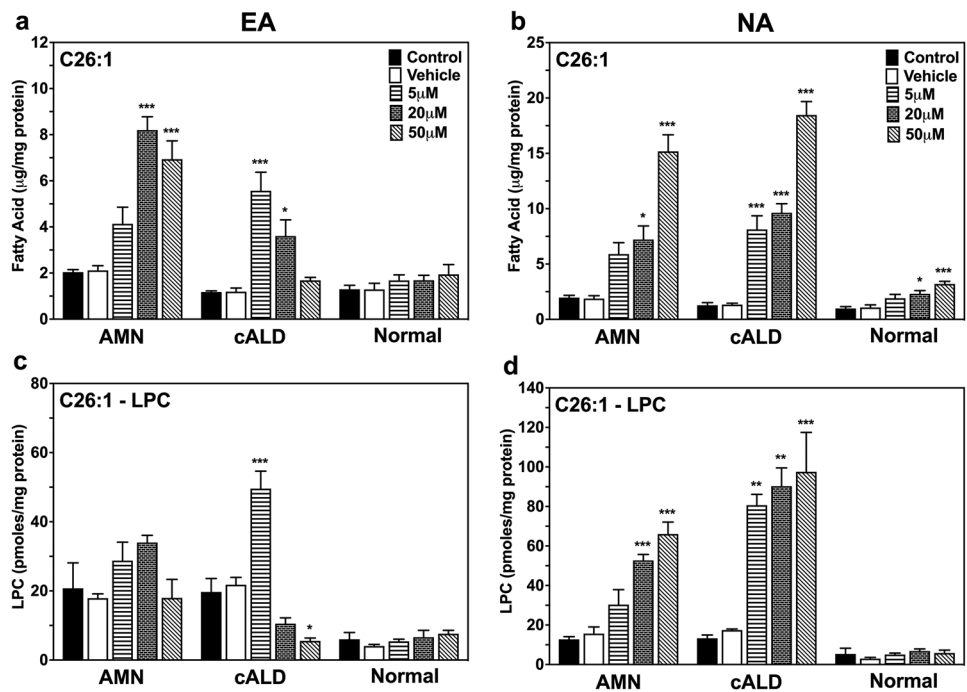

Discussion

Dietary therapy has been proven successful in managing metabolic disorders. For instance, a low-phenylalanine diet started immediately following diagnosis of phenylketonuria has been reported to keep blood phenylalanine levels in check and improve intelligence quotient and neuropsychological outcomes [23]. Similarly, a strict avoidance of fasting in addition to a fat-reduced and fat-modified diet is recommended in the management of long-chain fatty acid oxidation disorders [24]. To maintain normal plasma ammonia and amino acid concentrations in patients with urea cycle disorders, nutritional management is adopted that restricts dietary protein intake with adequate supply of protein-free energy, essential amino acid supplements, and vitamins and minerals in combination with

Fig. 5 Cytoprotective effects of nervonic acid (NA) against oxidative stress in ALD. AMN (GM17819), cALD (GM04904), and normal (NHDF) cells were pretreated with increasing concentrations of erucic acid (EA) or NA for 5 days and then stressed with hydrogen peroxide (H_2O_2, 200 µM) for 24 h. Cell viability (expressed as % viability of control cells) was measured using CyQUANT NF assay kit. Data from three independent experiments are shown as mean ± SEM. *$p < 0.05$ and **$p < 0.01$ show statistical significance from vehicle-treated cells using 2-tailed unpaired Student's t test
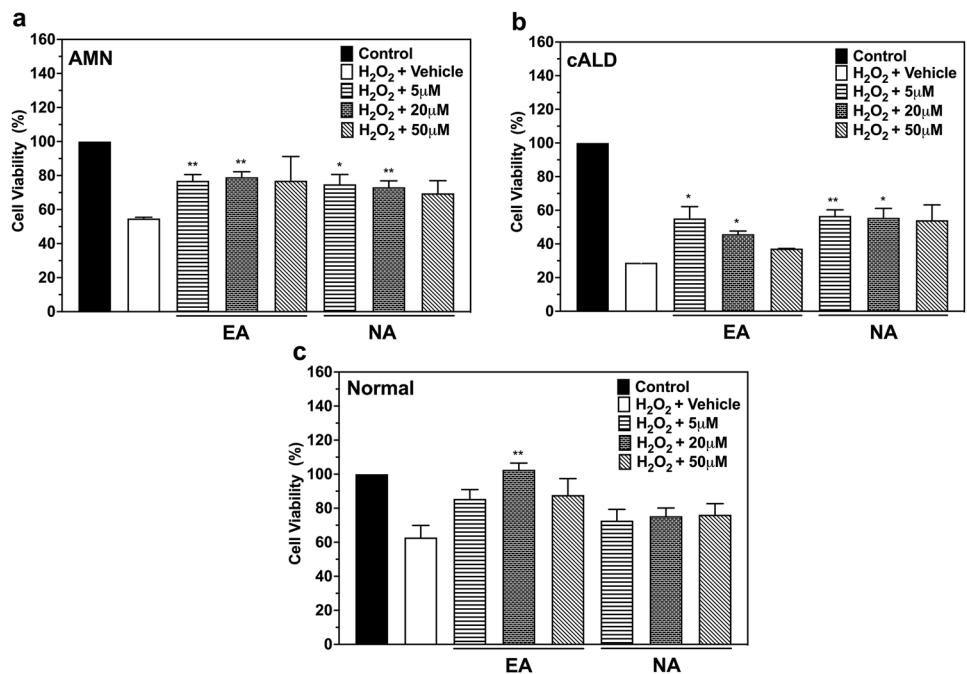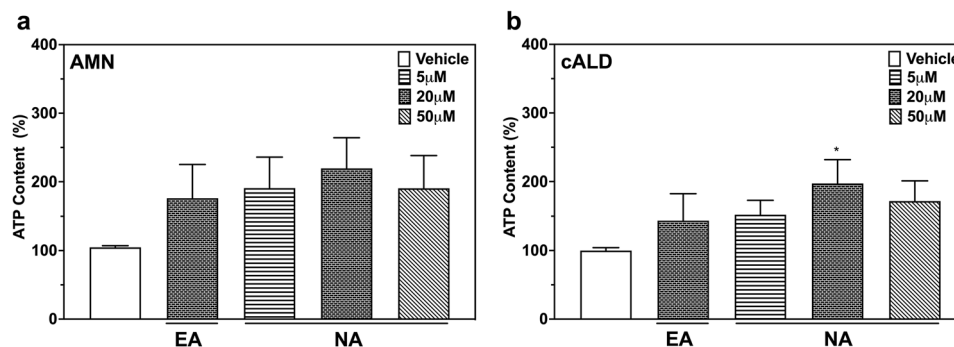
Fig. 6 Effect of nervonic acid (NA) on ATP production in ALD fibroblasts. AMN (GM17819) and cALD (GM04904) cells were pretreated with erucic acid (EA) or NA for 5 days. ATP levels was measured using ATPlite luminescence assay kit. Relative ATP content is shown as a percentage of vehicle-treated cells (considered 100%) after normalization with the CyQUANT NF assay values. Data from three independent experiments are shown as mean ± SEM. *$p < 0.05$ shows statistical significance from vehicle-treated cells using 2-tailed unpaired Student's t test

nitrogen-scavenging drugs [25]. Here, we demonstrate the benefits of using nervonic acid as a therapeutic strategy to delay and potentially arrest ALD disease progression. Following treatment with erucic acid, we observed a very strong negative correlation ($r = -0.9$) between C26:0 and C24:1 in both cALD and AMN fibroblasts indicating that increase in C24:1 very strongly and linearly corresponded with the decrease in C26:0 (Table 1). On the other hand, following nervonic acid treatment, AMN and cALD cells showed slightly variable strengths of association between C26:0 and C24:1. Further, this is the first study to elucidate the functional benefits of nervonic acid on ALD in addition to its biochemical effects. ALD is a genetic disorder that progressively affects the nervous system and adrenal glands. In the cerebral form of the disorder, it leads to symptoms such as cognitive deficits, blindness, and paralysis. At birth, ALD males and females are born with normal brain function and myelination occurs normally with no developmental delay; however, 35% of boys will develop this rapidly fatal demyelinating cerebral disease.

With the addition of ALD to newborn screening, it has become possible to diagnose ALD prior to symptom onset, offering time for early monitoring and treatment. These newborns receive comprehensive counseling and evaluations, which may contribute to improved outcomes [8, 9]. This is advantageous as ALD therapy is currently limited. However, detection of newborns with ALD places a heavy burden on families who may not have access to all available treatments due to eligibility or finances. HSCT may slow or stop the progression of the disease, but HSCT is most effective in individuals with early stages of the disease with evidence of cerebral involvement and no neurological symptoms [26]. It is not appropriate for boys prior to the development of cerebral disease detected on MRI. Besides HSCT, the only other treatment is adrenal hormone therapy for adrenal insufficiency. With few therapeutic options and potentially more patients in the future due to the expanded newborn screening, the development of safe and effective therapies that can arrest or delay disease progression is essential. Nervonic acid is one such option that can potentially limit the

Table 1 Correlation between cellular C26:0 and C24:1 or C22:1 following fatty acid treatments

Analytes	C26:0 vs C24:1	C26:0 vs C22:1	C26:0 vs C24:1	C26:0 vs C22:1
Treatment	**EA**		**NA**	
cALD (GM04904) cells				
Correlation Coefficient	−0.99	−0.59	−0.68	−0.26
Interpretation	Very strong negative correlation	Moderate negative correlation	Strong negative correlation	Weak negative correlation
AMN (GM17819) cells				
Correlation coefficient	−0.93	−0.69	−0.82	−0.52
Interpretation	Very strong negative correlation	Strong negative correlation	Very strong negative correlation	Moderate negative correlation

EA erucic acid (C22:1); *NA* nervonic acid (C24:1)

elevation of saturated VLCFA and the development of demyelinating lesions in asymptomatic ALD patients.

While the precise mechanism leading to cerebral disease is uncertain, there is strong evidence that the saturated VLCFA accumulation resulting from dysfunctional peroxisomal proteins induces cellular apoptosis and mitochondrial dysregulation and this leads to progressive demyelination [4, 6, 7, 27].

Nervonic acid can address the various pathological aspects of ALD [16, 28]. Nervonic acid is one of the monounsaturated fatty acids that occurs naturally in human milk and significantly increases during myelinogenesis in fetuses and infants and reflects brain maturity [29–32]. Nervonic acid can potentially support white matter development and prevent demyelination and may be used therapeutically in ALD. This is further supported by the decreased levels of nervonic acid in sphingomyelin observed in postmortem ALD brains, which has led to the notion that its biosynthesis is suppressed in this disease [15, 16]. As nervonic acid is important for myelination and is deficient in ALD, its supplementation could be beneficial for these patients. Furthermore, early administration of nervonic acid to asymptomatic ALD patients has the potential to delay the onset and progression of the various symptoms providing valuable time to intervene. Previous studies have examined the effects of other monounsaturated fatty acids such as erucic and oleic acids in ALD, but these primarily focused on their impact on saturated VLCFA accumulation [12, 13, 33, 34]. Nervonic acid is also a monounsaturated fatty acid that is enriched in sphingomyelin, a type of sphingolipid abundantly found in myelin sheaths and is catalyzed by ELOVL1 from erucic acid [4, 28, 31].

Limited studies have also considered the potential use of nervonic acid in demyelinating diseases, since its biosynthesis appears to be inhibited in ALD fibroblasts [15, 35]. However, none of these studies have examined the full potential of nervonic acid as a dietary therapy for ALD. The results of our study illustrate multiple prospective pharmacological effects of nervonic acid in patients with ALD. Nervonic acid can decrease saturated VLCFA accumulation, which is pathogenically prevalent in ALD. It can also enhance intracellular ATP production to avoid apoptosis and offer cytoprotection against oxidative stress resulting from excessive saturated VLCFA production, thus potentially preventing mitochondrial damage. Our study saw a greater reduction of C26:0 with erucic acid than nervonic acid, but the decrease in saturated VLCFA with nervonic acid was sufficient in eliciting cytoprotection and increase ATP levels. Similar functional benefits have been shown with palmitoleic acid (C16:1), an omega-7 monounsaturated fatty acid in adipocytes. It was shown to increase intracellular ATP production, indicating enhanced mitochondrial activity [36]. Also, palmitoleic acid demonstrated cytoprotective properties such as

preservation of cell proliferation and viability in pancreatic beta-cells [37].

Several studies have investigated the benefits of nervonic acid in various neurological disease models and have reported no serious adverse events [38, 39]. Recently, nervonic acid administered orally at 60 mg/kg in a Parkinson's disease (PD) mouse model showed potential for protecting the motor system without toxicity on the liver and kidney [40]. Nervonic acid also protected PC-12 cells from oxidative stress stimulated by 6-hydroxydopamine (6-OHDA), a cell model for PD [41]. Oral administration of mice with an oil-containing nervonic acid at ~23 mg/kg dose for 30 days caused improvement in learning and memory with no abnormal signs or death, suggesting that the long-term intake of nervonic acid is safe [42]. Similarly, mice fed with nervonic acid-supplemented diet (6 g/kg diet, 0.6%) for over 3 months did not show any toxicities including ruffled fur, anorexia, cachexia, skin tenting, skin ulcerations, diarrhea, or death. Additionally, this study also revealed that this diet improved energy metabolism in mice and thus may have potential use in the treatment of obesity and its associated complications [43].

Despite the beneficial outcomes of nervonic acid either alone or as part of the supplemented diets in preclinical models, few clinical studies have suggested a correlation between intake of omega-9 fatty acids including nervonic acid and an increased risk of cardiotoxicity in humans [44, 45]. In these studies, the authors have clearly indicated that their observations need to be interpreted cautiously, especially in determining the causality of the cardiotoxicity. In contrast, clinical studies have also shown nervonic acid to have preventive effects on obesity-related metabolic disorders [46]. This clearly indicates the need to better characterize dietary sources of monounsaturated fatty acids and understand nervonic acid pharmacology better and to optimize dosage regimens to minimize risks while increasing benefits.

Shifting of lipid metabolism through diet is an area of opportunity for ALD as this type of treatment is generally inexpensive and may be more convenient for patients. LO is a dietary mixture of erucic and oleic acids that has shown promise in normalizing C26:0 concentrations, but the association between the incidence of brain abnormalities and C26:0 concentrations is currently inconclusive [2, 11, 12, 33]. Despite LO reducing C26:0 levels in ALD, studies found little to no clinically beneficial effects in symptomatic patients and indeterminate results in asymptomatic patients [13, 47, 48]. This is due to many factors, but one of these is the limited information on erucic acid pharmacokinetics that could guide optimal dosage. In addition, the usage of erucic acid has been restricted due to lack of regulatory approval, and the risk of cardiotoxicity [5]. However, this adverse effect has been reported only in rodent studies and

myopathy secondary to the use of erucic acid, a component in mustard oil (e.g., seed oil from *Brassica nigra or Brassica juncea*), has not been reported either in populations ingesting it as cooking oils (as in China and India) or in patients taking LO. Nevertheless, the use of LO demonstrated potential competitive inhibition of ELOVL1 as a therapeutic strategy [33]. In ALD, the accumulated saturated VLCFA continues elongation by ELOVL1 [49, 50]. Erucic, oleic, and nervonic acids can act as substrates for ELOVL1, preventing C26:0 from further elongation and thus result in reduced saturated VLCFA levels and increased monounsaturated VLCFA [5, 50]. Similarly, we also observed an increase in C26:1 in all cells. However, it should be noted that till date, no known side effect has been reported because of an increase in monounsaturated fatty acids [2].

Using carefully designed, well-controlled studies, it is possible to develop a full pharmacological understanding of the therapeutic potential of nervonic acid, to identify concentrations that are beneficial with minimal side effects for future pre-clinical and clinical studies. Further investigation of nervonic acid as a potential and safe therapy for ALD is warranted. In vivo studies with nervonic acid will assess its biochemical and physiological effects. The safety, toxicity, and efficacy of nervonic acid will also be better evaluated in relevant animal models as it allows for the understanding of short-term and long-term exposure effects on blood and tissues such as the liver, heart, adrenals, spinal cord, and brain.

Disclosures

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