Evidence That Accumulation of Ceramides and Cholesterol Esters Mediates Oxidative Stress–Induced Death of Motor Neurons in Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is characterized by degeneration of motor neurons in the spinal cord resulting in progressive paralysis and death. The pathogenic mechanism of ALS is unknown but may involve increased oxidative stress, overactivation of glutamate receptors, and apoptosis. We report abnormalities in sphingolipid and cholesterol metabolism in the spinal cords of ALS patients and in a transgenic mouse model (Cu/ZnSOD mutant mice), which manifest increased levels of sphingomyelin, ceramides, and cholesterol esters; in the Cu/ZnSOD mutant mice, these abnormalities precede the clinical phenotype. In ALS patients and Cu/Zn-SOD mutant mice, increased oxidative stress occurs in association with the lipid alterations, and exposure of cultured motor neurons to oxidative stress increases the accumulation of sphingomyelin, ceramides, and cholesterol esters. Pharmacological inhibition of sphingolipid synthesis prevents accumulation of ceramides, sphingomyelin, and cholesterol esters and protects motor neurons against death induced by oxidative and excitotoxic insults. These findings suggest a pivotal role for altered sphingolipid metabolism in the pathogenesis of ALS.

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Amyotrophic lateral sclerosis (ALS) patients manifest progressive degeneration of motor neurons in the spinal cord and brainstem resulting in paralysis and death of the patients by respiratory failure.[1] Although the cause of most cases of ALS is unknown, a few families have been identified in which the disease is inherited in an autosomal dominant manner as the result of mutations in the antioxidant enzyme Cu/Zn-SOD.[2] Transgenic mice expressing the same Cu/Zn-SOD mutations exhibit histopathological and clinical phenotypes remarkably similar to ALS patients.[3–5] Data obtained from studies of patients, Cu/Zn-SOD mutant mice, and cultured neurons suggest that the pathogenic mechanism responsible for motor neuron degeneration involves oxidative stress,[6,7] overactivation of glutamate receptors,[8–10] and a form of programmed cell death called apoptosis.[11] Epidemiological findings suggest that dietary fat intake is associated with a higher risk of ALS, whereas diets high in fiber may decrease risk.[12]

Sphingolipids are a major class of membrane lipids in eukaryotic cells that are particularly abundant in the nervous systems of mammals. Sphingolipids are localized in plasma and endoplasmic reticulum membranes wherein they are concentrated, together with cholesterol, in microdomains called lipid rafts. Sphingolipid synthesis is initiated by serine palmitoyltransferase that catalyzes the condensation of palmitoyl-CoA with serine to form 3-dihydrosphinganine, which is converted to sphingosine and then acylated to form ceramide; choline then is added to ceramide to form sphingomyelin (Fig 1a). Originally thought to serve only a structural function in membranes, sphingomyelins are now recognized as serving complex signaling roles. One prominent group of signaling molecules that arise from de novo sphingomyelin synthesis and hydro-

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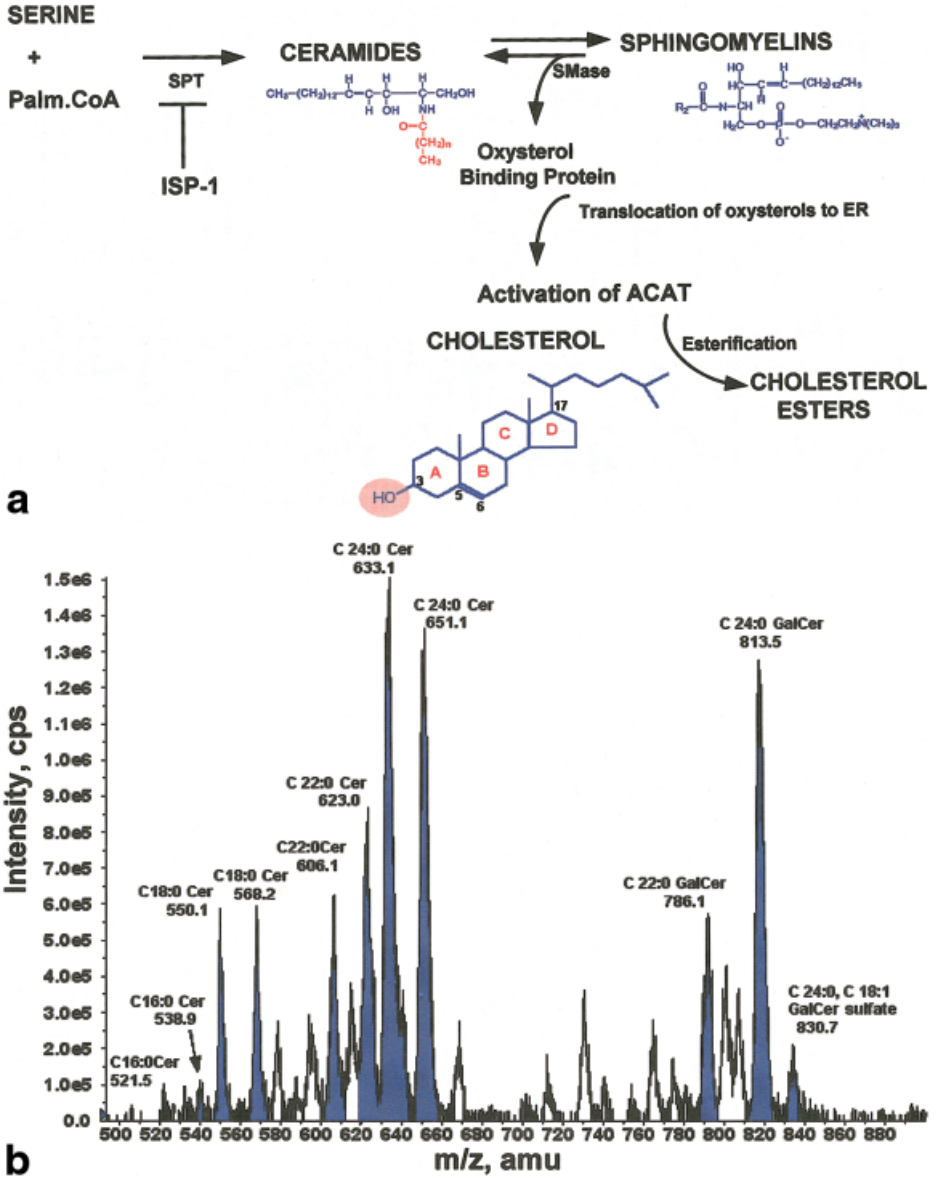


Fig 1. Metabolic pathways of sphingolipid and cholesterol metabolism, and identification of components of these pathways in spinal cord tissue. (a) Diagram showing pathways for production and metabolism of sphingomyelin and their relation to cholesterol metabolism. ACAT = acyl-coenzyme-A cholesterol actetyltransferase; SPT = serine palmitoyl transferase; SMase = sphingomyelinase. (b, [overleaf]) Electrospray ionization tandem mass spectrometry chromatograms showing ceramides (b) and cholesterol and cholesterol esters (c) in samples of human spinal cord tissue.

lysis is ceramides, which are generated in response to oxidative stress and by receptor-mediated activation of sphingomyelinases.[13,14] Ceramides may play important roles in regulating processes such as cell proliferation, differentiation, and programmed cell death[15] and have been implicated in the deaths of neurons that occur in ischemic stroke[16] and Parkinson's disease.[17] Activation of sphingomyelin hydrolysis also caused the dephosphorylation and release of oxysterol-binding protein into the cytosol where it binds and delivers oxysterols to the endoplasmic reticulum thereby activating acyl-coenzyme A: cholesterol acyltransferase and the formation of cholesterol esters (see Fig 1a). In this study, we document striking increases in levels of sphingomyelin, ceramides, and cholesterol esters in the spinal cords of ALS patients and transgenic mice expressing a familial ALS Cu/Zn-superoxide dismutase mutation and show that these abnormalities, which result from increased oxidative stress, can sensitize motorneurons to death.

Patients and Methods

Tissues from Human Control and Amyotrophic Lateral Sclerosis Patients

Fresh specimens of lumbar spinal cord from three neurologically normal and nine sporadic ALS patients were obtained at autopsy. Tissues were frozen immediately and stored at −80°C. The mean ages and standard deviations were 69 ± 14 and 58 ± 10 years, and the mean postmortem intervals and standard deviations were 10.4 ± 8.5 and 14.4 ± 4.9 hours for control and ALS patients, respectively. The differences between the means for either age or postmortem interval were not statistically significant between the two groups.

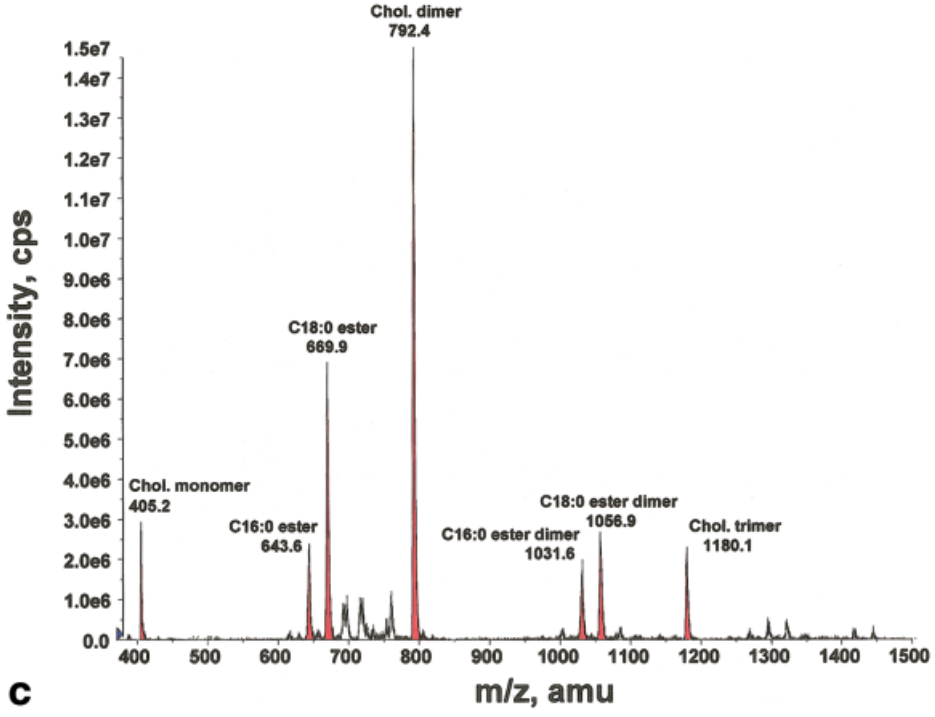


Figure 1 (Continued)

The causes of death in the control patients were pneumonia, prostate cancer, and acute myocardial infarction, and the cause of death in all ALS patients was respiratory failure.

Mice and Tissue Removal

Heterozygous breeding pairs of transgenic mice expressing the human Cu/Zn-SOD gene with a G93A mutation were purchased from Jackson Laboratories (Bar Harbor, ME). These mice were generated on a B6/SJL background. At weaning age, the offspring of the heterozygous matings were genotyped by reverse transcription polymerase chain reaction analysis. Heterozygote transgenic mice were used for these studies and became paralyzed in one or more limbs at 4 to 5 months of age; nontransgenic littermates were used as controls. Mice were killed with inhalation anesthesia, and whole spinal cords were removed, placed on dry ice, and stored at −80°C.

Cell Cultures, Experimental Treatments, and Analysis of Cell Survival

The NSC-19 cell line was generated by somatic cell fusion of mouse neuroblastoma N18TG2 cells with motor neuron-enriched spinal cord cultures from embryonic day 12 to 14 mice and were maintained at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and 50μg/ml of gentamicin.[18] Cells were subcultured by removing them from the substratum with squirts of medium; passages up to 30 were used. Primary cultures of mixed spinal cord cells were established from day 14 mouse embryos of B6/SJL mice as described previously.[9,10] Motor neurons were identified in the cultures as described in our previous studies.[10] The cultures were maintained at 37°C in a 5% CO_2 atmosphere in Neurobasal medium and B27 supplements (Life Technologies, Bethesda, MD). L-Glutamic acid and $FeSO_4$ were purchased from Sigma (St. Louis, MO), and stock solutions were prepared sterile water. 4-Hydroxynonenal was purchased from Cayman Chemicals (Ann Arbor, MI) and was stored as a concentrated stock in ethanol. Treatments were conducted in Locke's solution (154mM NaCl, 5.6mM KCl, 2.3mM $CaCl_2$, 1.0mM $MgCl_2$, 3.6mM $NaHCO_3$, 5mM glucose, 5mM HEPES, pH 7.2). Cells were grown in flasks to near confluence and pretreated with ISP-1 at 100pM for 1 hour, before adding palmitoyl-CoA, dimethoxynaphthoquinone (DMNQ), or hydrogen peroxide. Cells were allowed to incubate for 12 hours and then were scraped and centrifuged, and the lipids were extracted and the lipid profile was analyzed by electrospray mass spectrometry, mass spectrometry, (ES/MS/MS) as described above. For analysis of cell survival in NSC-19 cells, cells were plated onto 96-well plates at a density of 50,000 cells per well. Cells then were pretreated with ISP-1 at 100pM for 1 hour, before adding palmitoyl-CoA, DMNQ, or hydrogen peroxide. Cells were allowed to incubate for 48 hours, and the relative number of live cells was determined by using the XTT plus phenazine methosulfate assay (Sigma catalog no. X-4751); the plate well ultraviolet light absorbance was performed by a Perkin Elmer HTS 7000 Plus. To quantify the survival of motor neurons in primary spinal cord cultures, we exposed cells to experimental treatments for 24 hours and then stained them with the fluorescent DNA-binding dye Hoechst 33342. The percentage of motor neurons with condensed or fragmented nuclei was determined for each culture.

Lipid Extraction of Tissue and Cells

Total lipids from samples were prepared according to a modified Bligh and Dyer procedure.[19] Briefly, each sample was homogenized at room temperature in 10 volumes of deion-

ized water, then in 3 volumes of 100% methanol containing 30mM ammonium acetate, and vortexed. Four volumes of chloroform then were added, and the mixture was vortexed and then centrifuged at 1,000*g* for 10 minutes. The bottom (chloroform) layer was removed and analyzed by direct injection into a tandem mass spectrometer. Lipid extractions were performed using borosilicate-coated glass tubes, pipettes, and injectors.

Measurement of Sphingolipids, Phospholipids, Cholesterol Esters, and Lipid Peroxides

ES/MS/MS analyses were performed using methods similar to those used in previous studies.[20–23] Samples were injected using a Harvard Apparatus pump at 15μl/min into an electrospray ionization (ie, Turbo Ion Spray module) Sciex API 3,000 triple stage quadrupole tandem mass spectrometer (ES/MS/MS) from Sciex Inc. (Thornhill, Ontario, Canada) operated in the positive mode. The ion spray voltage (V) was 5,500 at a temperature of 80°C with a nebulizer gas of 8psi, curtain gas of 8psi, and the collision gas set at 4psi. The declustering potential was 80V, the focusing potential 400V, the entrance potential −10V, the collision energy 30V, and the collision cell exit potential was 18V. The MS/MS scanned from 300 to 2,000 atomic mass units (amu) per second at a step of 0.1amu. Each species of sphingolipids, phospholipids, cholesterol esters, and lipid peroxides initially was identified by a Q1 mass scan, then by precursor ion scanning or neutral loss scanning of a purified standard. Samples were injected into the ES/MS/MS for 3 minutes, where the mass counts accumulated and the sum of the total counts under each peak was used to quantitate each species. Sphingomyelins, ceramides, cholesterol, and cholesterol ester standards C16:0, C18:0, C18:1, and cholesteryl-arachidonate (C20:0) were purchased from Sigma. Ceramides C20:0, C24:0, C24:1, phosphatidylcholine C16:0–C18:1, C18:0–C18:1, phospatidylethanolamine C16:0–C18:1, phosphatidylglycerol C16:0–C18:1, phosphatidylserine C16:0–C18:1, phosphatidylinositol C16:0–C18:1, and phosphatidic acid C16:0–C18:1 were purchased from Avanti Polar Lipids (Alabaster, AL). Palmitoyl-lactosyl ceramide C16:0–C16:0, stearoyl-lactosyl-ceramide C16:0–C18:0, lignoceryl-glucosyl-ceramide C16:0–C24:0, lignoceryl-galactosyl-ceramide C16:0–C24:0, and stearoyl-galactosyl-ceramide-sulfate C18:1–C24:0 were purchased from Matreya Inc. (Pleasant Gap, PA). 4-hydroxynonenol and adducts were purchased from Cayman Chemicals.

Results

Levels of Sphingomyelin, Ceramides, and Cholesterol Esters Are Increased in Spinal Cords of Amyotrophic Lateral Sclerosis Patients and in Presymptomatic and Symptomatic Cu/Zn-SOD Mutant Mice

We used electrospray ionization (ES)/MS/MS) to identify ceramides, sphingomyelins, cholesterol esters, and phospholipids of various hydrocarbon chain lengths and double bond contents in spinal cord tissues from ALS patients and age-matched neurologically normal control patients (see Fig 1b and c). Quantitative comparisons of analyses performed on nine ALS and three control patients showed significant increases in the levels of ceramide C16:0, ceramide C24:0, and sphingomyelin in spinal cord tissue from ALS patients (Fig 2a). There was a small and nonsignificant increase in the level of C18:0 ceramide in ALS spinal cords. Levels of phospholipids were not different in samples from ALS and control patients (data not shown).

Transgenic mice overexpressing the G93A familial ALS Cu/Zn-SOD mutation exhibit progressive degeneration of motor neurons and associated hind limb paralysis.[3] In our mice, symptoms of hind limb paralysis became evident beginning at 16 to 20 weeks of age; the mice were killed within 2 to 3 weeks of disease onset. We quantified levels of sphingomyelin and ceramides in upper (cervical) and lower (lumbar) spinal cord tissue from end-stage disease Cu/ZnSOD mutant mice ($n = 8$), presymptomatic (12 week-old) mice ($n = 4$), and nontransgenic control mice ($n = 11$). Levels of sphingomyelin were significantly increased in the lower spinal cords of presymptomatic (20% increase; $p < 0.05$) and symptomatic (46% increase; $p < 0.01$) Cu/ZnSOD mutant mice compared with nontransgenic littermates. Levels of C16:0 ceramide were significantly increased in the lower spinal cords of presymptomatic and symptomatic Cu/ZnSOD mutant mice compared with nontransgenic mice. Levels of C24:0 ceramide and sphingomyelin were significantly increased in the lower spinal cords of symptomatic mice. There were no significant differences in the levels of C18:0 ceramide in the lower spinal cords of Cu/ZnSOD mutant mice compared with nontransgenic control mice. Levels of sphingomyelins and ceramides examined were unchanged in the upper spinal cords of Cu/ZnSOD mutant mice compared with nontransgenic mice (see Fig 2b). Collectively, these findings demonstrate alterations in sphinomyelin metabolism associated with selective vulnerability of lower spinal cord motor neurons in ALS patients and Cu/ZnSOD mutant mice.

In the spinal cords of ALS patients, levels of cholesterol esters C16:0 and C18:0 were drastically increased by more than 10-fold compared with spinal cords of control patients (see Fig 2c). Levels of cholesterol esters C16:0 and C18:0 in the lower spinal cords were each increased by more than fourfold in presymptomatic Cu/ZnSOD mutant mice, and by 10- to 15-fold in symptomatic Cu/ZnSOD mutant mice, compared with nontransgenic littermates (see Fig 2d). Levels of sphingomyelin, long-chain ceramides, and cholesterol esters were increased to a significantly lesser amount in the upper spinal cords of symptomatic Cu/ZnSOD mutant mice and were not yet increased in upper spinal cord tissue of presymptomatic mice (see Fig 2b and d), suggesting a specific change associated with the relative vulnerability of different spinal cord motor neurons at these two different time points.

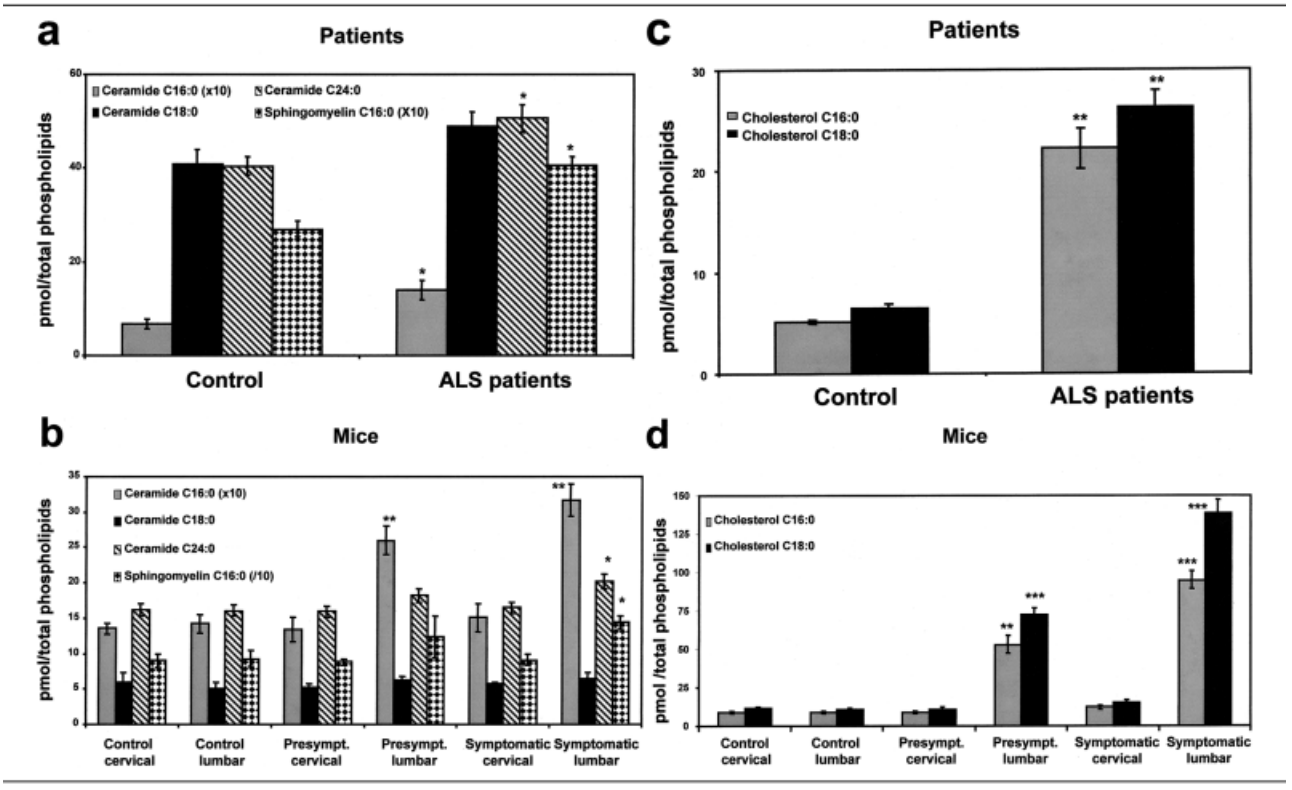


*Fig 2. Levels of ceramides and cholesterol esters are increased in spinal cord tissues of amyotrophic lateral sclerosis (ALS) patients and Cu/Zn-SOD mutant mice in a manner related to selective neuronal vulnerability and disease progression. Concentrations of ceramides and sphingomyelins (a, b) and of the indicated cholesterol esters (c, d) were quantified in spinal cord tissues from ALS patients and age-matched control patients (a, c) and in cervical and lumbar spinal cord samples from presymptomatic and symptomatic Cu/Zn-SOD mutant transgenic mice (b, d). Values are the mean and standard deviation of determinations made in samples from 9 ALS patients and 3 control patients, and in samples from 11 control, 4 presymptomatic, and 8 symptomatic Cu/Zn-SOD mutant mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with corresponding value for control human patients or control nontransgenic mice (analysis of variance with Scheffé post hoc tests).*

Levels of Ceramide and Cholesterol Esters Are Increased in Motor Neurons Exposed to Oxidative Stress by a Mechanism Requiring De Novo Sphingolipid Synthesis

Studies of nonneuronal cells have shown that oxidative stress can induce ceramide production[24,25] and accumulation of cholesterol esters.[26] Because it has been reported that levels oxidative stress, as assessed by measures of membrane lipid peroxidation and oxidative protein modifications, are increased in spinal cord cells of ALS patients and Cu/Zn-SOD mutant mice,[6,7] we sought to establish a cause–effect relationship between oxidative stress and increased levels of sphingomyelin and ceramides in ALS. The level of the lipid peroxidation product 2-pentylpyrrole, a lysine-4-hydroxynonenal conjugated adduct,[27] was drastically increased by more than sevenfold in spinal cord tissue of ALS patients compared with tissue from control patients (Fig 3a). Levels of 2-pentylpyrrole were also significantly increased in lumbar spinal cord tissue of presymptomatic (fourfold) and symptomatic (fivefold) Cu/Zn-SOD mutant mice compared with spinal cords of nontransgenic mice (see Fig 3b). Levels of 2-pentylpyrrole also were performed in samples of spinal cord from the cervical region of control, presymptomatic and symptomatic mutant-CuZnSOD mice. No significant differences in levels of 2-pentylpyrrole were observed in cervical spinal cord tissue between Cu/ZnSOD mutant mice and nontransgenic control mice (data not shown).

We next measured levels of sphingomyelin, ceramides, cholesterol esters, and 2-pentylpyrrole in NSC19 motor neuron-like cells exposed to agents that induce superoxide production (DMNQ)[28] or hydroxyl radical production (hydrogen peroxide).[29] The levels of C16:0 ceramide was significantly increased by 56% within 8 hours of exposure to DMNQ (Fig 4a). Levels of cholesterol esters were drastically increased, by fourfold in NSC19 cells within 8 hours of exposure to DMNQ (see Fig 4a). DMNQ and hydrogen peroxide had no significant effects on levels of sphingomyelin. Addition of palmitoyl-CoA (a substrate for serine palmitoyltransferase in the rate-limiting step of sphingolipid synthesis) to the culture medium resulted in significant increases in the levels

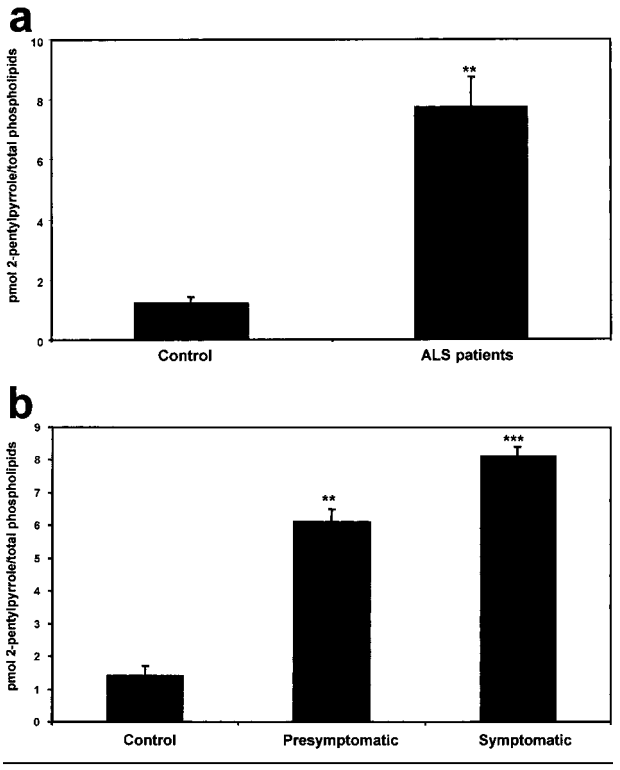
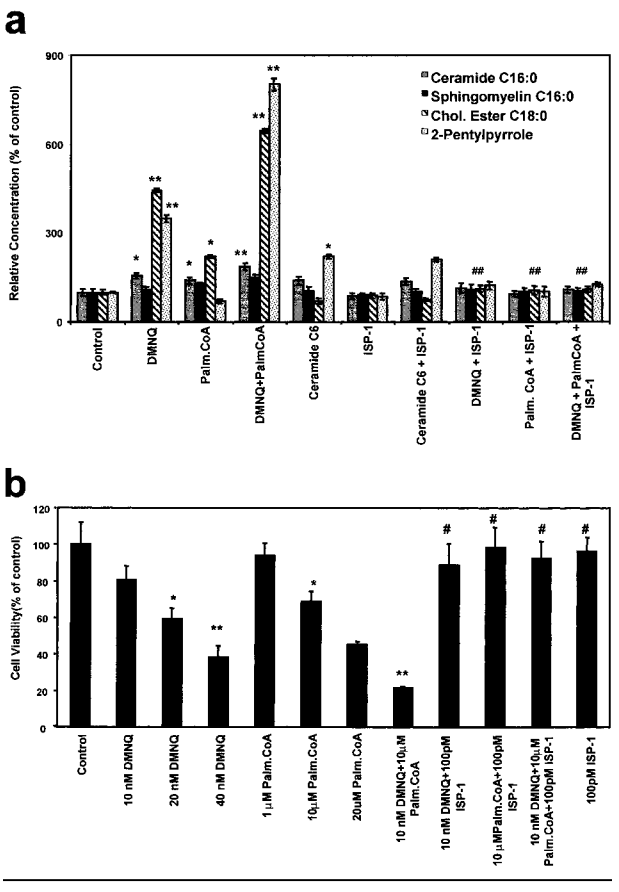


Fig 3. Membrane lipid peroxidation is increased in spinal cords of amyotrophic lateral sclerosis (ALS) patients and in Cu/Zn-SOD mutant mice before the appearance of motor dysfunction. Levels of 2-pentylpyrrole, an adduct containing the lipid peroxidation product 4-hydroxynonenal, were quantified in spinal cord tissues from ALS patients and age-matched control patients (a) and in lumbar spinal cord samples from presymptomatic and symptomatic Cu/Zn-SOD mutant transgenic mice (b). Values are the mean and standard deviation of determinations made in samples from 9 ALS patients and 3 control patients, and in samples from 11 control, 4 presymptomatic, and 8 symptomatic Cu/Zn-SOD mutant mice. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with corresponding value for control human patients or control nontransgenic mice (analysis of variance with Scheffé post hoc tests).

of sphingomyelin, ceramides, and cholesterol esters and potentiated the effects of DMNQ on the accumulations of ceramides and cholesterol esters. As expected, DMNQ increased levels of membrane lipid peroxidation, as indicated by large increases in the level of 2-pentylpyrrole; palmitoyl-CoA exacerbated the effects of DMNQ and hydrogen peroxide on membrane lipid peroxidation. To determine whether increased ceramide production might induce cholesterol ester accumulation, we exposed NSC19 cells to a membrane-permeant form of ceramide (C6-ceramide) and measured levels of cholesterol esters. However, levels of cholesterol esters did not increase in cells exposed to C6-ceramide (see Fig 4a).

To establish that the increased levels of ceramides and cholesterol esters induced by oxidative stress were the result of increased sphingomyelin metabolism, we blocked de novo synthesis of sphingolipids in NSC19 cells by treating them with ISP-1 (myriocin; an inhibitor of serine palmitoyltransferase, the rate-limiting step in sphingolipid synthesis).[30,31] In contrast with the

Fig 4. Sphingolipid synthesis and oxidative stress induce accumulation of ceramides and cholesterol esters and promote death of a motor neuron cell line. (a) NSC-19 motor neuron-like cells were exposed for 6 hours to 50nM DMNQ (an agent that induces superoxide production), 10μM plamitoyl-CoA, 1μM C6-ceramide, 100pM ISP-1, or the indicated combinations of treatments. Levels of ceramide C16:0, sphingomyelin, cholesterol esters C18:0, and 2-pentylpyrrole were quantified. Values are the mean and standard deviation of measurements made in cells from at least four different cultures. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with corresponding control value. #$p < 0.01$ compared with corresponding value for cells exposed to DMNQ, PalmCoA, or DMNQ+PalmCoA in the absence of ISP-1. (b) NSC-19 cells were exposed for 24 hours to the indicated concentrations of DMNQ or PalmCoA alone or in combination with 100pM ISP-1 as indicated and cell viability was quantified. Values are the mean and standard deviation of measurements made in cells from at least four different cultures. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control value. # $p < 0.01$ compared with corresponding value for cells exposed to DMNQ, PalmCoA, or DMNQ+PalmCoA in the absence of ISP-1 (analysis of variance with Scheffé post hoc tests).



large increases in levels of ceramides and cholesterol esters present in control NSC19 cells exposed to DMNQ, these oxidative insults caused only small increases in levels of ceramides and cholesterol esters in NSC19 cells pretreated with ISP-1 (see Fig 4a). Exposure of NSC-19 to hydrogen peroxide, an agent that induces hydroxyl radical production and membrane lipid peroxidation, also resulted in significant increases in levels of C16:0 ceramide and cholesterol esters, and these effects of hydrogen peroxide were prevented by treatment of cells with ISP-1 (data not shown).

Evidence That Sphingomyelin Metabolism Mediates Oxidative Stress–Induced Death of Motor Neurons

Because increased production of ceramides[32] and alterations in subcellular cholesterol metabolism[33] have been associated with apoptosis, we sought a causal role for increased sphingolipid metabolism in the death of motor neurons in ALS. DMNQ induced death of NSC19 cells in a concentration-dependent manner with 10, 20, and 40nM DMNQ killing 18, 39, and 60% of the cells, respectively. Palmitoyl-CoA also induced death of NSC19 cells with concentrations of 10 and 20μM killing 31 and 55% of the cells, respectively (Fig 4b). The vulnerability of NSC19 cells to DMNQ was significantly increased in cultures cotreated with palmitoyl-CoA, consistent with a role for sphingolipid metabolism in the cell death pathway activated by oxidative stress. Indeed, ISP-1 was very effective in protecting NSC19 cells against death induced by DMNQ, palmitoyl-CoA, and combined exposure to DMNQ and palmitoyl-CoA (see Fig 4b).

We next established primary dissociated cell cultures from spinal cords of embryonic mice; we previously showed that motor neurons in such cultures are vulnerable to excitotoxicity and to oxidative stress.[10] Because increased levels of extracellular glutamate and overactivation of glutamate receptors are believed to contribute to the death of motor neurons in ALS,[8] we assessed the involvement of sphingolipid metabolism in the excitotoxic process. Primary motor neurons were readily killed by glutamate (Fig 5). However, when cultures were pretreated with ISP-1 the motor neurons exhibited a highly significant increased resistance to excitotoxicity. The membrane lipid peroxidation product 4-hydroxynonenal is implicated in the death of motor neurons in ALS.[7,34] Primary motor neurons were killed by 4-hydroxynonenal, and ISP-1 pretreatment afforded significant protection against the toxicity of this aldehyde (see Fig 5).

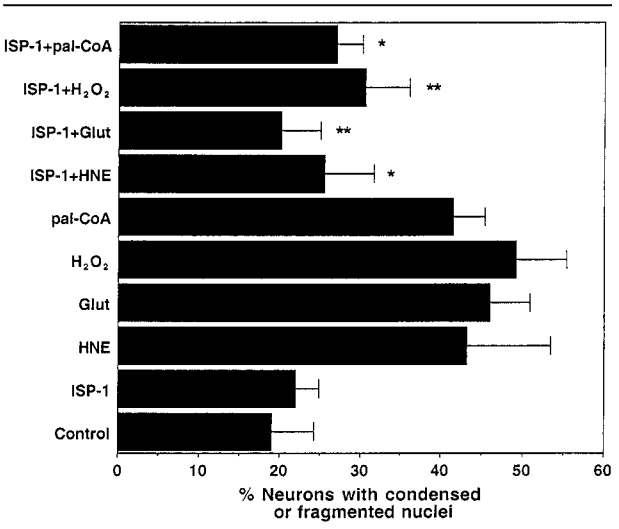


*Fig 5. Inhibition of serine palmitoyltransferase protects spinal cord motor neurons against excitotoxic cell death. Primary cultures of mouse spinal cord cells were exposed for 24 hours to the indicated treatments, and motor neuron survival was quantified (see Patients and Methods). Cells were pretreated with ISP-1 (50nM) for 1 hour before addition of other treatments. HNE = 1μM 4-hydroxynonenal; Glut = 20μM glutamate, H_2O_2 = 40 mm hydrogen peroxide; pal-CoA = 20μM palmitoyl CoA. Values are the mean and standard deviation of determinations made in four separate cultures. *p < 0.001, **p < 0.0001 compared with corresponding value for cultures not pretreated with ISP-1 (protected least significant difference (PLSD) analysis).*

Discussion

These findings document a profound abnormality in sphingolipid metabolism in ALS patients and Cu/Zn-SOD mutant mice and link this abnormality to motor neuron degeneration. The available data suggest a scenario for the pathogenesis of ALS as follows: genetic and/or environmental factors result in increased oxidative stress in motor neurons; the oxidative stress alters membrane sphingolipid metabolism in ways that increase the accumulation of long-chain ceramides and cholesterol esters; and the derangements of ceramides and cholesterol esters trigger cell death by rendering motor neurons vulnerable to excitotoxicity and oxidative stress. Because levels of ceramides and cholesterol esters were increased in the spinal cords of ALS mice at least 1 month before the appearance of clinical signs, it is unlikely that the increases were secondary to motor neuron degeneration. Our cell culture data confirm that oxidative stress can induce changes in ceramide and cholesterol esters similar to those seen in ALS patients and Cu/Zn-SOD mutant mice. Moreover, the ability of ISP-1 to prevent excitotoxicity and oxidative stress–induced death of motor neurons establishes a pivotal role for sphingolipid metabolism in death of motor neurons induced by oxidative stress and glutamate. We found that levels of 2-pentylpyrrole were significantly increased in spinal cords of ALS patients and presymptomatic Cu/ZnSOD mutant mice, consistent with previous data showing that oxidative stress is an early and pivotal event in the neurodegenerative pro-

cess in ALS.[6,34] Previous studies have documented increased free radical content in spinal cord but not brain of presymptomatic Cu/ZnSOD mutant mice.[35] We observed increased levels of oxidative stress and accumulation of ceramides and cholesterol esters in lower (but not upper) spinal cord tissue of presymptomatic Cu/ZnSOD mutant mice, consistent with a critical role for a selective oxidative stress burden in the most vulnerable population of spinal cord motor neurons.

We found that oxidative stress induces ceramide accumulation in motor neurons, consistent with previous studies showing that oxidative stress increases and antioxidants decrease ceramide levels in tumor cells.[36,37] When sphingolipid synthesis was reduced by treatment of cells with ISP-1, the production of ceramides and accumulation of cholesterol esters in response to oxidative stress were significantly decreased, demonstrating a requirement for sphingolipid metabolism in the generation of ceramides and altered cholesterol metabolism in motor neurons subjected to oxidative stress. Ceramides, in turn, can further increase oxidative stress by directly inhibiting mitochondrial complex III activity thereby increasing superoxide production.[38] When taken together with studies showing that inhibition of mitochondrial oxidative phosphorylation can render motor neurons vulnerable to excitotoxicity,[39] our data suggest a causal role for increased ceramide production in motor neuron degeneration in ALS. Recent findings suggest an important role for one or more ceramides in triggering apoptosis, a form of cell death implicated in ALS. In nonneuronal cells, ceramide analogs can induce caspase-3 activation and poly(ADP-ribose) polymerase cleavage,[40] and overexpression of the anti-apoptotic protein Bcl-2 can prevent ceramide-induced apoptosis.[31] Ceramides can also induce death of several different types of neurons in culture including hippocampal,[41] cortical,[42] mesencephalic,[17] and motor[43] neurons. Our data showing that ISP-1 protects motor neurons against oxidative and excitotoxic injury suggest a role for excessive ceramide production in motor neuron death in ALS. Sphingomyelin and ceramides may normally function as signaling molecules that mediate physiological responses to cytokines and neurotrophic factors[44] and adaptive responses to oxidative stress.[41] Indeed, mutations in serine palmitoyltransferase cause neuropathies in humans characterized by progressive degeneration of sensory neurons.[45] Furthermore, excessive production and/or accumulation of sphingolipids can be cytotoxic, as demonstrated in studies of sphingomyelinase-deficient mice[16] and mice overexpressing serine palmitoyltransferase.[46]

Our findings document a substantial increase in the levels of cholesterol esters in spinal cord cells of ALS patients and Cu/Zn-SOD mutant mice and in cultured motor neurons exposed to oxidative insults. Although these are the first data linking perturbed cholesterol metabolism resulting from oxidative stress in motor neurons to ALS, recent findings suggest roles for altered cholesterol metabolism in other age-related diseases. The pathogenesis of atherosclerosis involves oxidative stress, which causes the accumulation of sphingolipids that slow the efflux of sterols resulting in the accumulation of cholesterol esters and vascular cell damage.[47–49] Cholesterol esters also accumulate during renal tubular injury caused by oxidative stress and may contribute to acute renal failure.[26] Moreover, studies in which sphingomyelinases were manipulated indicate that ceramide production can induce accumulation of cholesterol esters.[50] A role for altered neuronal cholesterol metabolism in Alzheimer's disease is suggested by an association of increased levels of cholesterol esters with increased production of amyloid β-peptide in cultured cells[51] and by the ability of statins to decrease levels of amyloid β-peptide in vitro and in vivo.[52]

We propose, based on these findings and previous studies, that the neurodegenerative cascade in ALS involves an early increase in levels of oxidative stress, induced by genetic and/or environmental factors which causes a disturbance in membrane lipid metabolism resulting in the accumulation of ceramides and cholesterol esters. These findings in studies of cultured motor neurons and Cu/Zn-SOD mutant mice, when taken together with studies of nonneuronal cells showing that ceramide can mediate cell death induced by disturbances in plasma membrane redox systems,[51] suggest a pivotal role for disturbances of membrane lipid metabolism in the pathogenesis of ALS. The involvement of perturbed sphingolipid metabolism resulting in ceramide and cholesterol ester accumulation in motor neurons in ALS would suggest novel approaches for therapeutic intervention. We found that ISP-1, an inhibitor of serine palmitoyltransferase, was very effective in protecting motor neurons against excitotoxic and oxidative injury suggesting inhibitors of this enzyme as one class of potentially efficacious drugs. Agents that inhibit sphingomyelinases[16] or key enzymes in cholesterol ester formation,[52] and agents that decrease cholesterol levels,[53] are other examples of drugs that merit consideration for preclinical and clinical studies.

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