

Dysregulation of sphingolipid and sterol metabolism by ApoE4 in HIV dementia

R.G. Cutler, MS; N.J. Haughey, PhD; A. Tammara, BSc; J.C. McArthur, MD; A. Nath, MD; R. Reid, BA; D.L. Vargas, MD; C.A. Pardo, MD; and M.P. Mattson, PhD

Abstract—Background: Polymorphisms in apolipoprotein E have been associated with worse prognoses in numerous neurodegenerative conditions, including HIV dementia (HIVD). Despite these correlative observations, there has been little evidence suggesting a mechanism whereby the expression of ApoE4 renders neurons susceptible to insult. **Methods:** Electrospray ionization tandem mass spectrometry was used to quantify levels of sphingolipids and sterols in brains of HIVD patients. Data were stratified according to *APOE* genotype. **Results:** The authors found evidence of dysregulated lipid and sterol metabolism in HIVD patients with an *APOE4* genotype. They also found elevations of sphingomyelin, ceramide, and cholesterol in the medial frontal cortex, parietal cortex, and cerebellum of HIVD patients with an *APOE3/4* or *APOE4/4* genotype compared with HIVD patients with an *APOE3/3* genotype. There was no difference in the number of astrocytes or activated microglia in any brain region of the two patient populations, suggesting that modification of lipid metabolism in HIVD patients with an *APOE4* genotype was not the result of increased CNS inflammation. **Conclusions:** HIV dementia patients with an *APOE4* genotype may be sensitized to neural insults because of dysregulations in lipid metabolism.

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Neutral lipids are able to circulate in aqueous environments packaged as lipoproteins, a complex composed of phospholipid and a free cholesterol shell that surrounds a triglyceride and cholesterol-ester core. This complex is stabilized by surface apoproteins that function as ligands for lipoprotein receptors and cofactors for some enzymatic reactions. Apolipoprotein E is a component of several classes of lipoproteins, including chylomicron remnants, very low density lipoprotein (VLDL), and a subset of high density lipoprotein that regulate plasma lipid transport and clearance. In humans, ApoE is a 35-kDa protein that is composed of 299 amino acids. There are three major isoforms of ApoE that differ at two residues: ApoE2 has a cysteine at positions 112 and 158; ApoE3 has a cysteine at position 112 and an arginine at position 158; and ApoE4 has an arginine at positions 112 and 158. These single amino acid changes result in functional differences in ApoE isoforms, including their relative binding affinities for lipoproteins and ApoE receptors.¹

Apolipoprotein E is the most abundant apoprotein in the CSF and is produced in the brain where it serves paracrine-like functions. Astrocytes and mi-

croglia are the primary producers of ApoE, and receptors for apolipoproteins are abundant on neural cells. Neurons primarily express LRP, ApoE receptor 2, and VLDL receptor and thus are capable of incorporating lipoprotein complexes released from glia.²⁻⁴ ApoE-mediated lipid transport is necessary for normal neuronal function and promotes neurite outgrowth.^{5,6} An increasing body of evidence suggests that ApoE binding to its receptors may also serve important signaling functions that contribute to the correct placement of committed neurons during development,⁷ regulate nitric oxide and antioxidant production,^{8,9} and promote neurotrophic effects.¹⁰

The E4 allele of *APOE* has been determined to be a major risk factor for Alzheimer disease (AD)¹¹ and is associated with poor outcome after stroke,¹² cerebral hemorrhage,¹³ head trauma,¹⁴ and cardiac bypass.¹⁵ HIV-infected patients with the E4 isoform of *APOE* are more likely to have neurologic complications, including dementia and peripheral neuropathy,¹⁶ although an earlier study found no correlation between risk of HIV dementia (HIVD) and *APOE* genotype.¹⁷ The biophysical determinants of the E4 genotype that predispose neural tissue to malfunction and injury have not been

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From the Laboratory of Neurosciences (Drs. Haughey and Mattson, R.G. Cutler and A. Tammara), National Institute on Aging Gerontology Research Center, Baltimore, MD; and Departments of Neurology (Drs. Haughey, McArthur, Nath, Reid, Vargas, and Pardo), Pathology (Dr. Pardo), and Neuroscience (Dr. Mattson), Johns Hopkins University School of Medicine, Baltimore, MD.

R.G.C. and N.J.H. contributed equally to the work.

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Address correspondence and reprint requests to Dr. Norman J. Haughey, Department of Neurology, Johns Hopkins University School of Medicine, Meyer 6-109, 600 North Wolfe Street, Baltimore, MD 21287; e-mail: nhaughe1@jhmi.edu

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Table Numbers of glia in HIVD patients are not influenced by APOE genotype

	HLA-DR				GFAP			
	MFG-cc	MFG-wm	PAR	CBLM	MFG-cc	MFG-wm	PAR	CBLM
<i>APOE3</i>	3.2 ± 1.9	6.6 ± 1.9	3.6 ± 2.2	3.4 ± 1.0	2.9 ± 2.9	4.2 ± 2.4	3.5 ± 1.8	2.3 ± 0.9
<i>APOE4</i>	2.8 ± 2.6	5.0 ± 2.8	2.2 ± 1.4	3.0 ± .78	2.0 ± 0.8	3.6 ± 0.8	3.6 ± 0.7	2.7 ± 0.4
<i>p</i> Value	0.84	0.48	0.40	0.61	0.63	0.65	0.93	0.52

Reactive microglia (HLA-DR+) and astrocytes (GFAP+) were quantified in the cerebral cortex and white matter of the MFG (MFG-cc, MFG-wm), parietal cortex (PAR), and the cerebellum (CBLM) of HIV-infected patients with dementia (HIVD) by serology. There were no significant differences in the numbers of HLA-DR+ or GFAP+ cells in any brain region examined that were dependent on *APOE* genotype. Data are the area fraction immunopositive for the indicated antibody; absolute *p* values were determined using an analysis of variance with Tukey post hoc comparisons; n = 4 to 6 patients per group.

determined, although a role for the regulation of redox balance by ApoE has been suggested.^{8,18}

In an earlier study we found greater concentrations of sphingomyelin, ceramide peroxynitrite, and 4-hydroxynonenol in HIVD patients, suggesting that perturbation of sphingolipid metabolism may play a role in the initiation and progression of dementia in HIV-1-infected patients.¹⁹ Based on findings suggesting that ApoE polymorphisms can disrupt redox balance and evidence that redox balance modulates sphingolipid metabolism, we sought to determine whether *APOE* genotype affects concentrations of sphingomyelin, ceramide, and sterols in HIVD patients.

Materials and methods. *Human CSF samples and brain tissue.* Human brain tissue and CSF samples from the pre-highly active antiretroviral therapy era (pre-1996) were obtained from the Johns Hopkins AIDS brain bank. The brain tissues used in these studies have been well characterized and were selected for indicators of encephalitis using positive identification of macrophage infiltration and multinucleated giant cells and were free of opportunistic infections.¹⁹ Cognitive status was determined at least 6 months before death using the Memorial Sloan-Kettering (MSK) scale; at this time all patients had dementia and MSK scores >1.0. HIVD patients were categorized by *APOE* genotype into two groups: *APOE3/3* (n = 6) or *APOE3/4* and *4/4* (n = 4). All patients were black homosexual men of similar age (38 ± 5 years) with no history of IV drug use. Post mortem intervals were similar (25 ± 13 hours for *APOE3* patients and 15 ± 6 hours for *APOE4* patients), as were CD4 counts (40 ± 27 in *APOE3* patients and 33 ± 35 in *APOE4* patients).

Determination of APOE genotype. *APOE* genotypes were determined from snap-frozen autopsy tissue, including the spleen and liver. DNA was precipitated in a 1:10 volume of 3 mol/L NaAC and 2 volumes of cold 100% ethanol. After drying, DNA was resuspended in 6 to 8 μL ddH₂O. *APOE* was amplified by 40 cycles of PCR using the primer set U:TCCAAGGAGCTGCAGCGGCAGA and D:ACAGAATTCCGCCGCTG GTACACTGCCA. PCR products were digested overnight with *AflIII*, *HaeII*, and *HhaI* and separated by polyacrylamide gel electrophoresis, stained with ethidium bromide, and viewed by ultraviolet transillumination.

Immunohistochemical analyses. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex method. Paraffin sections were cut at 10 μm, mounted on slides, and incubated overnight at 37 °C. Slides were heated to 60 °C for 1 hour and dewaxed. For antigen retrieval, sections were either boiled in distilled water by a microwave oven for 15 minutes (for glial fibrillary acidic protein [GFAP]) or boiled in citrate buffer for 15 minutes (for HLA-DR). Nonspecific binding was blocked using goat (GFAP) or horse serum (HLA-DR) in phosphate-buffered saline (PBS) containing 0.4% Triton X-100 for 1 hour at room temperature. GFAP and HLA-DR antibodies were used at a dilution of 1:100, and biotinylated antirabbit and antimouse secondary antibodies were used at 1:200 (Vector Laboratories, Burlingame,

CA). Slides were washed with PBS and incubated for 1 hour in the presence of streptavidin-peroxidase complex. Sections were washed in water and then incubated for 5 minutes in the presence of diaminobenzidine and counterstained with cresyl violet. We quantified the amount of immunoreactivity for GFAP and HLA-DR using the fractional area method as described previously.¹⁹ In brief, using a light microscope interfaced with a Stereo Investigator System (MicroBrightfield, Inc., Williston, VT), the software selects a random starting site and provides a systematic sampling of the outlined field that is divided randomly in 100 μm × 100 μm squares, and at each selected site it projects a counting frame (130 μm × 100 μm) with a superimposed counting frame composed of small equidistant crosses. On each counting frame, all the crosses that coincide with nucleated immunoreactive cells are counted. The sum of immunoreactive points for all counting frames divided by the total number of crosses is the fractional area. This procedure is then repeated for each antibody. The investigator conducting the measurements was blinded to the diagnostic categories.

Lipid extraction and measurements of sphingolipids, phospholipids, and lipid peroxides. Total lipids from samples were prepared according to a modified Bligh and Dyer procedure.²⁰ Briefly, each sample was homogenized at room temperature in 10 volumes of deionized water, then in 3 volumes of 100% methanol containing 30 mmol/L ammonium acetate, and vortexed. Four volumes of chloroform then were added, and the mixture was vortexed and then centrifuged at 1000g 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. Electrospray ionization tandem mass spectrometry (ESI/MS/MS) analyses were performed using methods similar to those used in our previous studies.¹⁹ Samples were injected for 3 minutes, allowing for accumulation of mass counts, and the sum of the total counts under each peak was used to quantify each species. Sphingomyelin and ceramide standards C16:0 and C18:0 were purchased from Sigma (St. Louis, MO). Ceramides C20:0 and C24:0, 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). Standards for cholesterol and the cholesterol esters C16:0 and C18:0 were obtained from Sigma.

Results. *APOE4 increases the concentrations of sphingolipids and sterols in HIVD patients.* We quantified the number of GFAP+ astrocytes and HLA-DR+ microglia in the frontal cortex, parietal cortex, and cerebellum of HIVD patients. There were no differences in the numbers of GFAP+ or HLA-DR+ cells in any of the brain regions sampled of patients with *APOE4* compared with patients with *APOE3* (table).

We stratified HIVD patients according to *APOE* genotype and quantified levels of sphingolipids and sterols in three brain regions by ESI/MS/MS. In all the brain regions we examined, HIVD patients with the E4 allele of *APOE*

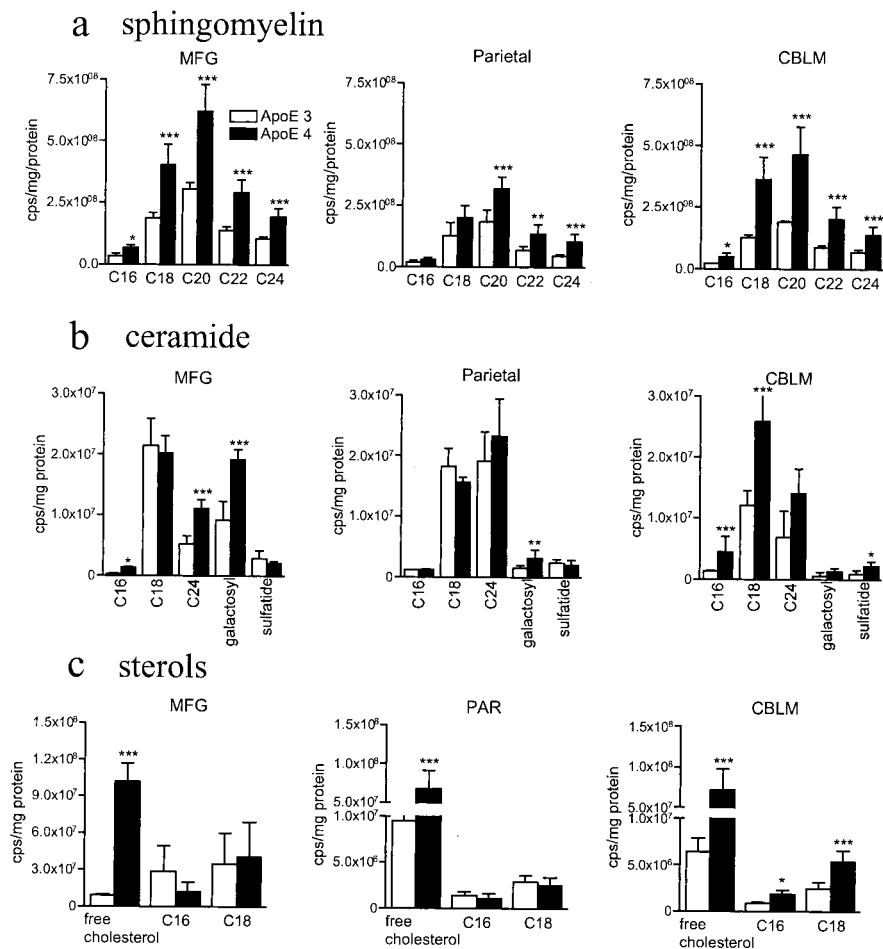


Figure. Sphingomyelin (A), ceramide (B), and sterols (C) are increased in HIV-infected patients with dementia (HIVD) with APOE4 genotypes. Sphingomyelin, ceramide, free cholesterol, and cholesterol esters were quantified by tandem mass spectrometry using reference standards to identify sphingomyelin C16, C18, C20, C22, and C24, ceramide C16, C18, and C24, galactosyl, sulfatide, cholesterol, and the cholesterol esters C16 and C18. In the medial frontal gyrus (MFG) and cerebellum (CBLM), there were significant increases in all forms of sphingomyelin in patients with an APOE4 genotype compared with patients with an APOE3 genotype. In the parietal cortex (PAR), there were significant increases in sphingomyelin C20, C22, and C24 in patients with APOE4 compared with patients with an APOE3 genotype. Ceramides C16 and C24 and galactosyl were significantly increased in the MFG of patients with APOE4 vs APOE3. In PAR, galactosyl was increased in patients with APOE4, and in the CBLM ceramides C16 and C18 and sulfatide were significantly increased in patients with an APOE4 genotype compared with patients with an APOE3 genotype. In the MFG, PAR, and CBLM, the concentrations of free cholesterol were significantly increased

*in patients with an APOE4 vs APOE3 genotype. In CBLM, the cholesterol esters C16 and C18 were significantly increased in patients with the APOE4 allele. Analysis of variance with Tukey post hoc comparisons. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; $n = 4$ to 6 patients per group.*

had greater concentrations of sphingolipids and sterols compared with HIVD patients with APOE3. There were increases of sphingomyelin C16, C18, C20, C22, and C24 in the medial frontal gyrus (MFG), C20 in the parietal cortex, and concentrations of C16, C18, C20, C22, and C24 in the cerebellum of HIVD patients with an APOE4 genotype compared with HIVD patients with an APOE3 genotype (figure, A). The ceramides C16 and C24 and galactosyl were increased in the MFG; galactosyl was increased in the parietal cortex; and C16, C18, and sulfatide were greater in the cerebellum of patients with an APOE4 genotype compared with patients with an APOE3 genotype (figure, B). Free cholesterol was increased in the MFG and in the parietal cortex. In the cerebellum, free cholesterol and the cholesterol esters C16 and C18 were increased in HIVD patients with the E4 allele of APOE compared with HIVD patients with the E3 allele (figure, C).

Discussion. The APOE4 genotype is associated with a faster rate of cognitive decline in AD, with a worse prognosis after traumatic and ischemic insult and is associated with a higher rate of neurologic complications in patients with HIV.^{11-14,16} Despite these correlative observations, there has been little biochemical evidence linking the ApoE haplotype to

an increased sensitivity of neural cells to dysfunction or death. We have previously shown that sphingolipids are increased in HIVD¹⁹ and now present findings that HIVD patients with an APOE4 genotype, compared with HIVD patients with an APOE3 genotype, have greater concentrations of some sphingolipids and sterols in brain tissues. These observations suggest that in an APOE4 genetic background, neurons may be sensitized to insult by dysfunctions in lipid metabolism. Although inflammatory products can modify lipid metabolism and promote sphingomyelin and ceramide synthesis, there was no evidence of increased CNS inflammation in HIVD patients with an APOE4 genotype compared with APOE3, suggesting that the APOE haplotype directly influences cellular lipid metabolism.

Normal neuronal function and neurite outgrowth require ApoE-mediated delivery of lipids.^{5,6} Moreover, neuronal repair mechanisms involve the salvage and recycling of lipids that allow for the sprouting of neurites and synaptogenesis.²¹ Thus, an inadequate delivery of lipoproteins to neurons could disrupt the architecture and function of neuronal membranes and render neurons susceptible to insult.

As evidence, AD patients with *APOE4* genotype show a more rapid decline of cognitive function than patients with an *APOE3* genotype, suggesting a more rapid deterioration of neuronal cells.²² Although there is no evidence yet available for the effects of an *APOE4* genotype on the progression of HIVD, some intriguing preliminary studies suggest that the presence of at least one *APOE4* allele may be associated with a greater risk of HIVD in older patients.²³

Sphingomyelins and ceramides are important constituents of specialized signaling domains known as lipid rafts.²⁴ At the plasma membrane, these domains anchor and consolidate receptors and signal transduction machinery into microdomains that serve to amplify and direct signal transduction. Perturbation of sphingolipid metabolism can disrupt the structure of lipid rafts and thereby disorganize receptor and signal transduction mechanisms and increase the vulnerability of cells to stress. As evidence, manipulations that increase sphingomyelin synthesis have been shown to sensitize neuronal cells to oxidative damage.²⁵ In a previous study, we found that sphingomyelin levels were increased in the MFG of patients with HIVD concurrent with increases of the lipid peroxidation product 4-hydroxynonenal.¹⁹ In the present study, we present evidence that sphingomyelin levels can be further increased in HIVD patients with an *APOE4* genotype. Therefore, it is likely that neural cells in HIVD patients with an *APOE4* genotype are more sensitive to oxidative stress because of increased sphingomyelin synthesis.

Lipids in planar membranes are distributed in an organized and segregated manner that is maintained by the actions of phospholipid translocases.²⁶ Sphingomyelin is predominantly located in the outer leaflet of the planar membrane. An early event in apoptosis is the loss of membrane asymmetry, during which sphingomyelin is brought into contact with the cytosol. Sphingomyelinases that are associated with the inner leaflet of the plasma membrane convert it to phosphatidylcholine and ceramide. Ceramide is involved in numerous cellular processes, including cell differentiation, proliferation, survival, and apoptosis. Ceramide can increase the permeability of the outer mitochondrial membrane to proteins of up to 60 kDa, including cytochrome *c*,²⁷ and disrupt mitochondrial respiration.²⁸ Mitochondrial toxicity is thought to contribute to neuronal dysfunction and death in HIVD,²⁹ and recent findings have shown that HIV-1 protein-induced mitochondrial membrane depolarization is exaggerated in human neural cultures with *APOE4* genotypes.³⁰ Our findings of excessive sphingomyelin accumulation in the brains of HIVD patients with an *APOE4* genotype suggest that a dysfunction in sphingolipid metabolism may be responsible for the sensitivity of neuronal mitochondria to HIV-associated insult in an *APOE4* genetic background. A potential trigger for this process may be a disruption of calcium ho-

meostasis. An overabundance of cytosolic calcium can promote lipid scrambling and ceramide production³¹ and is thought to be an important contributor to neuronal demise in HIVD.³²

Alterations in serum cholesterol are common in HIV-infected patients. HIV infection can decrease circulating cholesterol levels in association with immune activation³³ and increases in anticholesterol antibodies.³⁴ Protease treatment decreases circulating levels of anticholesterol antibodies³⁵ and averts decreases in serum cholesterol but can result in lipodystrophy and hyperlipidemia^{36,37} with consequent increases in the risk for diabetes and cardiovascular disease.^{37,38} In contradistinction to low serum cholesterol, we observed dramatic increases in free and esterified cholesterol in MFG, parietal cortex, and cerebellum. These alterations in cholesterol homeostasis are reminiscent of deficient cholesterol transport and storage that accompanies mutations in the Niemann–Pick C gene. Clinically, Niemann–Pick disease causes neurodegeneration that is characterized by the presence of Alzheimer-type neurofibrillary tangles composed of hyperphosphorylated tau. Neurons hyperimmunopositive for τ -2, neurofibrillary tangle-like structures, and elevated levels of tau in CSF have been described in HIV-infected patients and are associated with short life expectancy.³⁹⁻⁴¹ Cholesterol metabolism is intimately associated with the phosphorylation state of tau, neurite growth, and synaptic structure. Dystrophic neurites and synaptic simplification have been reported in HIVD, but the phosphorylation status of tau has not been addressed. The relationship between cholesterol, tau, and neurodegeneration suggests that neurofilament pathology may be pronounced in some cases of HIVD, although this remains to be tested.

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