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Oxidative stress in HIV demented patients and protection ex vivo with novel antioxidants

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reached statistical significance. L-deprenyl, didox, imidate, diosgenin, and ebselen blocked the CSF-induced toxicity. No effect of trimidox, ruthenium red, or Quercetin was seen.

Conclusions: Increased oxidative stress is present in brain and CSF of HIV-infected patients. There is also an accumulation of toxic substances in the CSF that are capable of inducing oxidative stress. The authors have identified several novel compounds that are capable of blocking the CSF-induced toxicity, the therapeutic potential of which is worthy of further exploration.

Much attention has been given recently to oxidative stress in the setting of HIV infection. Serum lactate levels are frequently elevated,[1] suggesting mitochondrial dysfunction; however, the role of oxidative stress in HIV pathogenesis remains uncertain. It also remains unknown whether oxidative stress plays a role in the pathogenesis of HIV dementia. Certainly oxidative stress seems to play an important role in the pathogenesis of other neurodegenerative diseases[2,3] and antioxidants may alter the course of progression of AD.

Recognition of dementia as an important consequence of HIV infection and the realization of its socioeconomic impact has led to the establishment of large multicenter research groups to evaluate the efficacy of antiretroviral or neuroprotective drugs in this patient population. However, the rationa0 T 7in.

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HIV infection without encephalitis (two patients had minor cognitive impairment, three were normal), and five with no HIV infection (three patients died of bacterial sepsis, one of cirrhosis, and one of pneumonia) were stained as described below. Postmortem intervals varied between 6 and 17 hours. All groups were age matched with a range of 29 to 57 years. All human tissue samples were obtained from the national neuro-AIDS tissue consortium. Brain tissue samples were obtained from three macaques infected with a chimeric strain of HIV and simian immune deficiency virus (SHIV) that developed encephalitis. An uninfected macaque was used as a control. Five micron thick, paraffin-embedded, formalin-fixed sections from the temporal lobe and basal ganglia were immunostained with a well-characterized monoclonal anti-4-hydroxynonenal (HNE)/protein complex antibody 1g4h7 (1:100).[5] A standard immunohistochemistry protocol was used as described previously.[6]

CSF samples. All CSF samples were centrifuged and cell free CSF was aliquoted and immediately frozen at -70 °C until further testing. Severity of dementia was categorized in the HIV-infected patients using the Memorial Sloan Kettering Scale (MSK). The patients were divided into three groups: no dementia (MSK = 0; n = 16), mild dementia (MSK = 0.5 or 1; n = 22), and moderate to severe dementia (MSK = 2 or 3; n = 8). CSF from patients with headaches or degenerative disc disease (n = 11) or MS (n = 9) were used as controls. The former group is referred to as normal controls. CD4 cell counts and details of antiretroviral therapy were available on all patients. CD4 cell counts (cells/mm^3; mean ± SEM) were as follows: nondemented group, 262 ± 48; mildly demented group, 164 ± 28; moderately to severely demented group, 125 ± 51. Despite a trend for decrease in CD4

dot-blotting technique. After the transfer, membranes were blocked with 3% BSA (in phosphate buffered saline [PBS] with 0.01% sodium azide and 0.2% Tween-20) overnight at 2 to 8 °C. The nitrocellulose membrane was exposed to a primary rabbit anti-DNPH protein antibody from ONCOR Oxyblot (1:150 working dilution) for 1 hour, and then to a secondary antibody (antirabbit immunoglobulin [Ig]G coupled to alkaline phosphatase [Sigma, St. Louis, MO]) diluted in the blocking solution 1:15,000 for 90 minutes at room temperature. Membranes were washed after every step in washing buffer (PBS with 0.01% sodium azide and 0.2% Tween-20). The nitrocellulose paper was then developed by a Sigmafast tablet, dissolved in 10 mL of distilled water, until the bands of oxidized proteins changed color. Blots were analyzed using computer-assisted scion imaging software.

Cultures of human brain cells. Brain specimens were obtained from human fetuses of 12 to 14 weeks gestational age with consent from women undergoing elective termination of pregnancy and approval by the University of Kentucky Institutional Review Board. Neuronal cultures were prepared as described previously.[8,9] Briefly, the cells were mechanically dissociated; suspended in Opti-MEM (Gibco, Gaithersburg, MD) with 5% heat-inactivated fetal bovine serum, 0.2% N2 supplement (Gibco, Gaithersburg, MD), and 1% antibiotic solution (penicillin G 10^4 units/mL, streptomycin 10 mg/mL, and amphotericin B 25 µg/mL); and plated in flat bottom 96-well plates. The cells were maintained in culture for at least 1 month before conducting the mitochondrial experiments.

Measurement of mitochondrial membrane potential activity. At the time of experimental treatment, the culture medium was replaced with Locke buffer containing (in mM) 154 NaCl, 5.6 KCl, 2.3 $CaCl_2$, 1 $MgCl_2$, 3.6 $NaHCO_3$, 5 glucose, and 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.2), and neurons were incubated with either known mitochondrial toxins (3-nitroproionic acid [3NP], staurosporine, or valinomycin) or CSF from each patient. Initially CSF samples (eight nondemented patients, and six each with either mild dementia, moderate to severe dementia, or MS, or normal controls) were analyzed by serial dilutions and we determined that 1:100 dilution was sufficient to show mitochondrial toxicity. No toxicity was noted in the headache or MS groups even at 1:3 dilution. Hence for the remainder of the study we used all CSF samples at 1:100 dilution in Locke buffer. CSF from patients with moderate to severe dementia was also analyzed with or without L-deprenyl (N,N-dialkylated amphetamine, N-methyl-N-propargylamphetamine; 1 µM), didox (3,4-dihydroxybenzohydroxanic acid; 100 µM), trimidox (3,4,5-tetrahydroxybenzohydroxamide; 100 µM),

solution.

JC-1 is a fluorescent dye that measures changes in mitochondrial membrane potential.[10] It exists as a green fluorescent monomer at low membrane potential. Once loaded into the mitochondria, JC-1 aggregates in regions of high potential, giving a red florescence. The resulting shift of the dye is used to detect changes in mitochondrial activity. Optical measurements were acquired with excitation at 485 nm and emission at 527 nm and 590 nm. The levels of fluorescence at both emission wavelengths were quantified and ratio of measurements was assessed. The data were calculated as mean ± SEM for mean optical measurements from three

Detection of oxidized lipids in brain. Prominent staining for HNE was noted in neurons, glial cells, and perivascular cells of patients with HIV encephalitis (figure 1). Interestingly, even in patients who died of bacterial sepsis, only small numbers of cells immunostained for HNE, indicating that comparatively there are massive amounts of oxidative stress in patients with HIV encephalitis (see figure 1).
Immune reactive cells for HNE were also present in all animals with SHIV encephalitis. No HNE-positive cells were present in the uninfected animal. Large numbers of neurons and glia were immunoreactive for HNE. Intense immunoreactivit

from all groups of patients and normal controls. As shown in figure 3, oxidized proteins were elevated in both mild dementia ($p < 0.001$) and moderate to severe dementia ($p < 0.01$) groups when compared to normal controls.

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Figure 3. Oxidative stress in brain and CSF. Protein oxidation in CSF of HIV-infected patients. CSF samples were analyzed by slot blot using antisera for protein carbonyl production. Increased protein carbonyl formation is noted in the CSF of patients with mild HIV dementia (MD) when compared to HIV-infected patients without dementia (ND) (**$p < 0.001$) and normal controls (NC) ($p < 0.05$). Patients with moderate to severe (Mod-S) HIV dementia also had elevated protein carbonyl levels compared to normal controls (*$p < 0.05$).

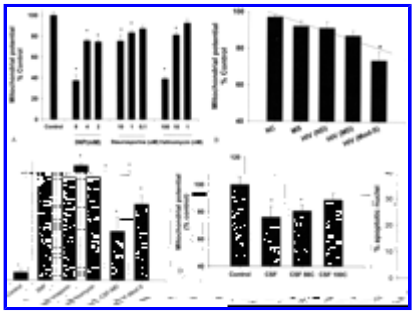

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Figure 4. Neurotoxic properties of CSF. (A) Effect of mitochondrial toxins on human fetal neurons. Cultures of human fetal neurons were treated with 3NP, staurosporine, or valinomycin and mitochondrial activity determined by JC-1 assay. A significant decrease in mitochondrial potential in a dose responsive manner was seen with each of the compounds. Data represent mean ± SEM. *$p < 0.05$. (B) Effect of CSF on mitochondrial membrane potential. Human fetal neurons in culture were treated with CSF and mitochondrial activity determined by JC-1 assay. A progressive decrease in mitochondrial membrane potential is seen with CSF from the groups of patients with HIV infection (HIV [ND] = no dementia; HIV [MD] = mild dementia; HIV [Mod-S] = moderate to severe dementia) when compared to patients with MS or normal controls (NC). Data represent mean ± SEM. *$p < 0.01$. (C) Induction of apoptosis by CSF and mitochondrial toxins. Significant neuronal apoptosis was induced by all three mitochondrial toxins (staurosporine, valinomycin, and 3NP) as well as CSF samples from patients with mild dementia or moderate to severe dementia. *$p < 0.05$ When compared to controls. Data represent mean ± SEM. (D) Effect of heat treatment of CSF on mitochondrial potential. Human fetal neurons were exposed to CSF from patients with moderate to severe HIV dementia following heat treatment at 56 °C or 100 °C. Partial reversal of toxicity is noted. Data represent mean ± SEM. *$p < 0.05$.

Effect of mitochondrial toxins on human fetal neurons. To characterize the susceptibility of human fetal neurons to mitochondrial injury, we initially treated cultures of human fetal neurons with three known

mitochondrial toxins—3NP, staurosporine, and valinomycin—each of which causes mitochondrial toxicity by a different mechanism

Effect of antioxidants on CSF-induced mitochondrial toxicity. R(-)-deprenyl (1 to 1,000 μM), diosgenin (2, 10 μM), ebselen (1, 5 μM), ruthenium red (1 to 100 μM), quercetin (5 to 50 μM), and trolox (1 nM to 200 μM), when tested alone, did not produce any significant changes in mitochondrial potential in neurons. Didox (≤100 μM), trimidox (≤200 μM), and imidate (≤50 μM) were also nontoxic but at higher concentrations produced decreases in mitochondrial potential (data not shown).

We next determined whether these drugs could block the CSF-induced mitochondrial toxicity. These experiments were conducted only with CSF samples from the moderate to severe dementia group. L-deprenyl (1 μM), didox (100 μM), imidate (1 μM), diosgenin (10 μM), ruthenium red (100 μM), quercetin (25 μM), and ebselen (5 μM) blocked CSF-induced neurotoxicity. No effect of trimidox (100 μM) was seen (figure 6). Trolox (10 μM) was used as a positive control that blocked CSF-induced neurotoxicity completely, suggesting that the neurotoxicity is predominantly via induction of oxidative stress.

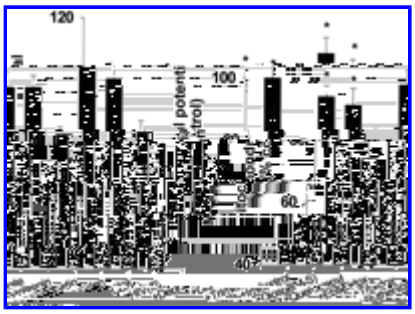

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Figure 6. Effect of novel antioxidants on CSF-induced mitochondrial dysfunction. Human fetal neurons were exposed to L-deprenyl (1 μM), didox (100 μM), imidate (1 μM), diosgenin (10 μM), Euk8 (500 μM), ebselen (5 μM), trimidox (100 μM), or trolox (10 μM) followed by CSF from HIV-infected patients with moderate to severe dementia. All pharmaceutical agents except trimidox were able to block the CSF neurotoxicity. Data represent mean ± SEM. *$p < 0.05$.

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We demonstrate the presence of oxidative stress in brain and CSF of patients with HIV dementia and that circulating toxins in CSF can further induce mitochondrial damage. This mitochondrial damage can lead to release of cytochrome *c* and initiate a cascade of events leading to apoptosis. Polyunsaturated fatty acids, which make up the brain's membrane phospholipids, are especially vulnerable to free radical attack because their double bonds allow easy removal of an H atom from an allylic carbon. Oxidation of polyunsaturated fatty acids results in the production of multiple aldehydes with different carbon chain lengths including HNE.[11] HNE forms adducts with proteins by covalent bonding to histidine, lysine, and cysteine residues.[12] HNE is toxic to neurons and astrocytes by causing disruption of a variety of cell surface receptors and transport mechanisms.[2] Here, using immunohistochemical techniques, we demonstrate that increased levels of HNE were present in macaques with SHIV

encephalitis. Factors that lead to the production of HNE in HIV-infected patients still need to be characterized; however, we have previously shown that the HIV envelope protein gp120 can cause lipid peroxidation as measured by thiobarbituric acid reactive substances.[12]

Protein carbonyl analysis is used as a general assay for oxidative damage to proteins. Carbonyl derivatives are formed by reactive oxygen species mediated oxidation of side chains of some amino acid residues. Carbonyl groups also may be introduced into proteins by glycoxidation and lipid peroxidation products.[13] The oxidation of proteins by free radicals may be responsible for damaging enzymes critical in neuronal function.[14]

such as Tat and gp120, various cytokines, quinolinic acid, glutamate, and other uncharacterized substances.[4]

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