model of the wound signal transduction pathway, in which JA and ethylene are both required for *pin* gene expression (Fig. 5). On wounding, ethylene regulates endogenous JA levels, and application of exogenous JA induces ethylene biosynthesis, which is required to induce a positive effect. As yet we cannot discriminate between parallel events in which wounding induces a small rise in JA, together with a rise in ethylene which triggers an additional rise in JA, and sequential events in which the wound-induced small increase in JA causes ethylene synthesis and its action in turn further amplifies the JA signal.

Jasmonates are much discussed currently for their importance as wound, abiotic stress, and developmental signals (*26*). At least during the wound response, ethylene and JA influence each other's levels in the plant and together act to regulate *pin* gene expression. It will be interesting to determine how many other effects of JA and related fatty acids require ethylene action.

REFERENCES AND NOTES

1. C. A. Ryan, *Plant Mol. Biol.* **19**, 123 (1992).
2. T. R. Green and C. A. Ryan, *Science* **175**, 776 (1972).
3. P. D. Bishop, D. J. Makus, G. Pearce, C. A. Ryan, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3536 (1981).
4. G. Pearce, D. Strydom, S. Johnson, C. A. Ryan, *Science* **253**, 895 (1991).
5. E. E. Farmer and C. A. Ryan, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7713 (1990).
6. H. M. Doherty, R. R. Selvendran, D. J. Bowles, *Physiol. Mol. Plant Pathol.* **33**, 377 (1988).
7. H. Peña Cortes, T. Albrecht, S. Prat, E. W. Weiler, L. Willmitzer, *Planta* **191**, 123 (1993).
8. S. H. Doares, J. Narvaez Vasquez, A. Conconi, C. A. Ryan, *Plant Physiol.* **108**, 1741 (1995).
9. E. E. Farmer and C. A. Ryan, *Plant Cell* **4**, 129 (1992); S. H. Doares, T. Syrovets, E. W. Weiler, C. A. Ryan, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4095 (1995).
10. H. Kende, *Annu. Rev. Plant Physiol.* **44**, 283 (1993).
11. S. F. Yang and N. E. Hoffman, *Annu. Rev. Plant Physiol.* **35**, 155 (1984).
12. J. E. Lincoln *et al.*, *J. Biol. Chem.* **268**, 19422 (1993); D. C. Olson, J. A. White, L. Edelman, R. N. Harkins, H. Kende, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5340 (1991); W. H. Rottmann *et al.*, *J. Mol. Biol.* **222**, 937 (1991); N. Nakagawa, H. Mori, K. Yamazaki, H. Imaseki, *Plant Cell Physiol.* **32**, 1153 (1991); W. K. Yip, T. Moore, S. F. Yang, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2475 (1992); C. S. Barry *et al.*, *Plant J.* **9**, 525 (1996).
13. C. A. Ryan, *Plant Physiol.* **54**, 328 (1974); A. Kernan and R. W. Thornburg, *Plant Physiol.* **91**, 73 (1989).
14. G. Felix and T. Boller, *Plant J.* **7**, 381 (1995); F. Greulich, T. Yoshihara, A. Ichihara, *J. Plant Physiol.* **147**, 359 (1995).
15. A. Schaller, D. R. Bergey, C. A. Ryan, *Plant Cell* **7**, 1893 (1995).
16. A. D. M. Glass and J. Dunlop, *Plant Physiol.* **54**, 855 (1974).
17. H. Veen, "Use of inhibitors of ethylene action," in *Manipulation of Ethylene Responses in Horticulture*, M. S. Reid, Ed., *Acta Hort.* **201**, 213 (1987).
18. E. C. Sisler, S. M. Blankenship, M. Guest, *Plant Growth Regul.* **9**, 157 (1990).
19. H. C. Yen, S. Y. Lee, S. D. Tanksley, M. B. Lanahan, H. J. Klee, J. J. Giovannoni, *Plant Physiol.* **107**, 1343 (1995).
20. A. J. Hamilton, G. W. Lycett, D. Grierson, *Nature* **346**, 284 (1990).
21. H. Peña Cortes, S. Prat, R. Atzorn, C. Wasternack, L. Willmitzer, *Planta* **198**, 447 (1996).
22. E. E. Farmer, D. Caldelari, G. Pearce, K. Walker Simmons, C. A. Ryan, *Plant Physiol.* **106**, 337 (1994).
23. E. Bell, R. A. Creelman, J. E. Mullet, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8675 (1995).
24. S. Ghosh and E. Kopp, *Science* **270**, 2018 (1995); M. Ruffer, B. Steipe, M. H. Zenk, *FEBS Lett.* **377**, 175 (1995).
25. For quantification of endogenous jasmonic acid, 2 g of leaf material was used for each assay. Quantification was performed by an enzyme-linked immunosorbent assay according to Lehmann *et al.*, *Planta* **187**, 156 (1995).
26. G. Sembdner and B. Parthier, *Annu. Rev. Plant Physiol.* **44**, 569 (1993).
27. Twenty micrograms of total RNA per slot was fractionated by electrophoresis on denaturing agarose gels, and blotted to Hybond-N (Amersham). Complementary DNA probes were prepared by the random priming method as described by J. Sambrook, E. F. Fritsch, and T. Maniatis [*Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)] using a [α-^{32}P]-dATP. Hybridization was performed at 42°C in 50% formamide, 5× SSC, 10× Denhardts solution, 6% PEG 8000, 12.5 mM $NaPO_4$ (pH 6.5), and 100 mg/ml denatured salmon sperm. DNA blots were washed once in 2× SSC/0.1% SDS and twice in 0.2× SSC/0.1% SDS at 42°C and exposed to XAR film (Xograph) with an intensifying screen at −80°C.
28. Total RNA was isolated from approximately 1 g of frozen tissue as described by J. Logemann *et al.* [*Anal. Biochem.* **163**, 16 (1987)].
29. J. S. Graham *et al.*, *J. Biol. Chem.* **260**, 6561 (1985).
30. S. C. Peck and H. Kende, *Plant Mol. Biol.* **28**, 293 (1995).

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Immunologic NO Synthase: Elevation in Severe AIDS Dementia and Induction by HIV-1 gp41

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Indirect mechanisms are implicated in the pathogenesis of the dementia associated with human immunodeficiency virus–type 1 (HIV-1) infection. Proinflammatory molecules such as tumor necrosis factor α and eicosanoids are elevated in the central nervous system of patients with HIV-1–related dementia. Nitric oxide (NO) is a potential mediator of neuronal injury, because cytokines may activate the immunologic (type II) isoform of NO synthase (iNOS). The levels of iNOS in severe HIV-1–associated dementia coincided with increased expression of the HIV-1 coat protein gp41. Furthermore, gp41 induced iNOS in primary cultures of mixed rat neuronal and glial cells and killed neurons through a NO-dependent mechanism. Thus, gp41-induced NO formation may contribute to the severe cognitive dysfunction associated with HIV-1 infection.

Neurocognitive deficits are common in HIV-1 infection. Twenty to 30% of patients with acquired immunodeficiency syndrome (AIDS) develop dementia during the course of their illness (*1*). HIV-1 frequently enters the central nervous system (CNS) early in the course of infection and replicates particularly in cells of macrophage origin, including microglia and perivascular macrophages (*2*, *3*). In human brain tissue, HIV-1 has occasionally been detected in astrocytes but rarely if ever in neurons (*2*–*4*). Despite the lack of productive HIV-1 infection in neurons, there is modest neuronal loss in the cortex as well as synaptic loss and dendritic simplification (*5*). The pathological changes of myelin pallor and breakdown of the blood-brain barrier are associated with HIV-1 dementia (*5*, *6*). However, the degree of neuropathologic change may not parallel the severity of neurological symptoms (*6*–*8*), and thus indirect mechanisms are most likely to be involved in the pathogenesis of AIDS (*9*).

Recent studies suggest a possible association between HIV-1 infection of macrophages and the severity of dementia (*2*, *10*). The HIV-1 coat protein gp120, which is shed by the virus, could contribute to neuronal cell death by excitotoxic mechanisms through activation of the *N*-methyl-D-as-

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partate (NMDA) glutamate receptor or through voltage-dependent Ca^{2+} channels (*11*). Neuronally derived nitric oxide (NO) may contribute to gp120 toxicity because inhibitors of NO synthase (NOS) prevent neurotoxicity (*12*). NO can kill cells through both necrotic and apoptotic pathways—acute high levels of NO produce killing through necrosis and chronic low levels of NO produce predominantly apoptotic features (*13*). The recent demonstration of potential apoptotic mechanisms mediating neuronal injury in simian immunodeficiency virus (SIV) encephalitis (*14*) as well as HIV-1 encephalitis (*15*) is consistent with chronic NO exposure. In glial cultures, HIV-1 and its associated coat proteins gp160 and gp41 induce the expression of proinflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin-1β, platelet-activating factor, and eicosanoids, as well as immunologic (type II) NOS (iNOS) (*16–18*). Some of these proinflammatory molecules damage neurons and oligodendrocytes in culture systems and thus may play a role in AIDS dementia (*19, 20*).

To study changes in iNOS expression associated with AIDS dementia, cortical brain tissue samples were obtained from the AIDS Brain Bank at the Johns Hopkins Hospital in Baltimore, Maryland. Tissue from a total of 29 patients was studied (*21*). Twenty-five patients were HIV-1 seropositive and were diagnosed with AIDS before death, whereas four patients were HIV-1 seronegative. Of the 25 patients with AIDS, 8 had no cognitive impairment and 17 had dementia, as determined by neurological and neuropsychological assessment, the severity of which was categorized with the Memorial Sloan Kettering (MSK) criteria (*22*). Cognitive dysfunction was mild (MSK 1 or 2) in 10 cases and severe (MSK 3 or 4) in 8 cases.

Reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out as a semiquantitative analysis for human iNOS detection, with expression of the the human β-actin gene used as an endogenous internal standard (*23*). Uncompromised amplification of iNOS cDNA and comparable amplification kinetics for both iNOS and β-actin sequences were achieved by addition of β-actin–specific primers after 10 cycles of iNOS cDNA amplification. Kinetic analysis showed that under these conditions, iNOS and β-actin cDNA amplification occurred in the linear range at 35 cycles and 25 cycles, respectively, ensuring an accurate comparison of iNOS mRNA to β-actin mRNA ratios (*24*).

In the brain tissue of AIDS patients without dementia or with mild dementia (MSK 1 or 2), there was a modest increase in the expression of human iNOS as compared with that in seronegative controls, which was not statistically significant (Fig. 1). The brain tissue of AIDS patients with severe dementia (MSK 3 or 4) showed a sixfold increase in human iNOS mRNA expression as compared with that in seronegative controls ($P \leq 0.0002$). Nucleotide sequence analysis of the iNOS PCR products confirmed the amplification of authentic human iNOS (*25*). The RT-PCR assay used in the current study for detection of human iNOS mRNA was more sensitive than that used in our previous study (*23*). As determined by serial dilutions of cloned human iNOS plasmid DNA (*26*), we were able to detect as few as 10 copies of human iNOS mRNA (*24*). This increased

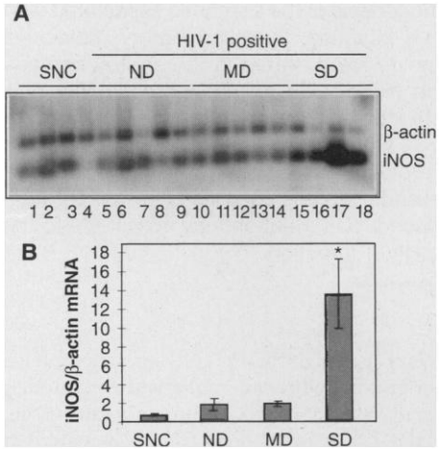

Fig. 1. Detection and kinetic analysis of iNOS and β-actin mRNA expression. (**A**) Human iNOS mRNA levels (35 PCR cycles) as compared with β-actin mRNA levels (25 PCR cycles) in cortical tissue obtained at autopsy from HIV-1 seronegative controls (SNC, lanes 1 through 4) and from HIV-1–infected patients with no dementia (ND, lanes 5 through 9), mild dementia (MSK 1 or 2) (MD, lanes 10 through 14), or severe dementia (MSK 3 or 4) (SD, lanes 15 through 18). These results were replicated three times with similar results (*23*). (**B**) Mean levels of human iNOS mRNA relative to levels of β-actin mRNA from HIV-1 SNCs and from HIV-1–infected patients with ND, MD, or SD. The levels of iNOS mRNA relative to those of β-actin were analyzed for significance by means of the Kruskal-Wallis test for multiple groups ($P \leq 0.0099$). For Fisher's protected least significance difference post hoc test, the comparisons of SNC to SD, ND to SD, and MD to SD were significant at $P \leq 0.0002$, $P \leq 0.0002$, and $P \leq 0.0003$, respectively. Data are means ± SEM.

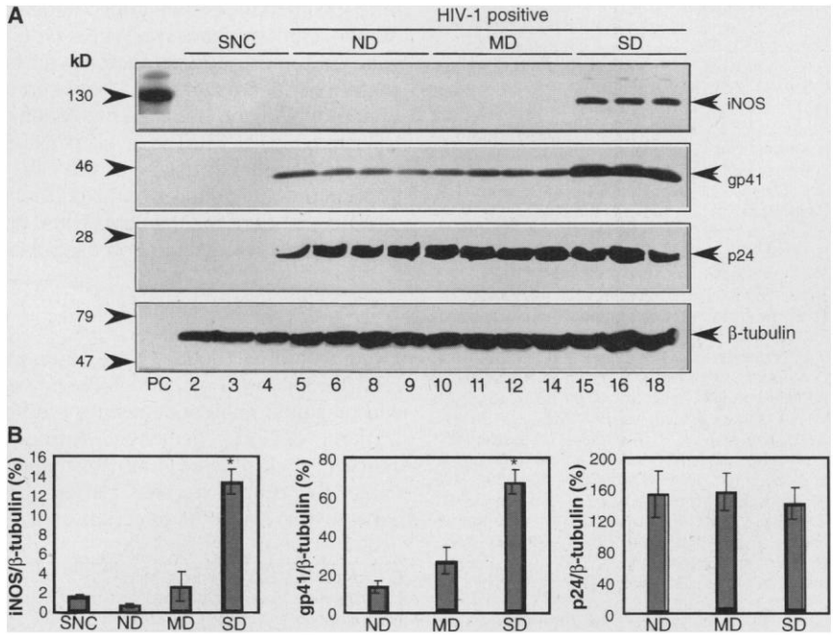

Fig. 2. Expression of iNOS protein in SD coincides with increased gp41 expression. (**A**) iNOS, gp41, p24, and β-tubulin protein immunoblots of postmortem cortical tissue from HIV-1 SNCs and from HIV-1–infected patients with ND, MD (MSK 1 or 2), or SD (MSK 3 or 4). Lack of availability of tissue for case numbers 1, 7, 13, and 17 prevented their inclusion in the protein immunoblot analysis. These results were replicated three times with similar results (*27*). (**B**) Mean levels of iNOS protein, gp41, and p24 relative to that of β-tubulin from HIV-1 SNCs and from HIV-1–infected patients with ND, MD (MSK 1 or 2), or SD (MSK 3 or 4). Representative blots are shown in (A). Eleven additional cases were included in the statistical analysis (*29*). The levels of iNOS, gp41, and p24 were analyzed for significance by means of the Kruskal-Wallis test for multiple groups: $P \leq 0.00012$, $P \leq 0.0009$, and $P \leq 0.6009$, respectively. The Spearman rank correlation test was used to compare iNOS/β-tubulin ratios to gp41/β-tubulin and p24/β-tubulin ratios and yielded ρ 0.721, $P \leq 0.0007$ and ρ 0.334, $P \leq 0.1172$, respectively (*29*). Fisher's least significance difference posthoc test indicated highly significant differences for iNOS ($P \leq 0.0001$) for SNCs to SD, ND to SD, and MD to SD. For gp41, Fisher's test indicated highly significant differences for ND to SD and MD to SD ($P \leq 0.0001$). Data are means ± SEM.

sensitivity probably accounts for our ability to detect human iNOS mRNA expression in control patients, which contrasts with our inability to detect human iNOS transcripts in the previous analysis (*23*). These findings indicate that human iNOS mRNA may be constitutively expressed at very low levels in "normal" postmortem human brain tissue.

We assessed the expression of iNOS protein by protein immunoblot analysis, using β-tubulin levels as an internal standard to control for differences in protein degradation and postmortem interval (*27*). Expression of iNOS protein in the cortical brain tissue of AIDS patients coincided with the levels of iNOS mRNA. The iNOS protein was increased eightfold in the cortex of patients with severe (MSK 3 or 4) HIV-1 dementia, as compared with the level in seronegative controls ($P \leq 0.0001$) (Fig. 2).

Because HIV-1 coat proteins induce iNOS in in vitro culture systems (*17, 18*), we wondered whether the coat proteins gp41 and gp120, which result from the cleavage of gp160, would be similarly elevated in AIDS patients with severe dementia (MSK 3 or 4). We accordingly assessed the expression of gp41 and gp120 by protein immunoblot analysis (*27*), using monoclonal antibodies directed against gp41 and gp120, and compared their levels with that of HIV-1 nucleocapsid phosphoprotein 24 (p24). As in previous reports (*28*), we were unable to detect gp120 (*29*). There was an elevation of gp41 protein ($P \leq 0.0001$), which was statistically associated with the expression of iNOS in patients with severe dementia ($P \leq 0.0007$) (Fig. 2), whereas p24 levels were not elevated in the HIV-1–infected patients (Fig. 2). β-tubulin levels were essentially the same in all the cases, confirming the specificity of the elevation of iNOS and gp41 (Fig. 2).

To ascertain whether the induction of iNOS by gp41 mediates neuronal cell death, we used a primary culture system of mixed rat neuronal and glia cells and assessed cell viability (*30*) (Fig. 3). Mixed neuronal-glial cultures were used because iNOS-induced neurotoxicity requires the presence of glia (*19*). The gp41 (100 nM) protein induced iNOS in mixed neuronal cultures after 7 days of continuous exposure (Fig. 3), whereas 10 nM gp41 was without effect (*31*). Thus, there appears to be a critical level of gp41 that is required for iNOS induction, which is consistent with our observations in human tissue. Neuronal cell death occurred through NO formation, as the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) provided neuroprotection that was reversed by excess substrate, L-arginine (L-Arg) (Fig. 3). gp41 neurotoxicity appears to require glia because toxicity was not observed in pure neuronal cultures (*31*). To control for possible nonspecific induction of iNOS and toxicity, we examined the effects of other glycoproteins: HIV-1 gp120 and p24 and the human T cell lymphocytotropic virus 1 (HTLV-1) glycoprotein 46 (gp46), which were prepared in a similar recombinant system for iNOS induction and neurotoxicity. As was consistent with previous reports, gp120 did not elevate iNOS in rodent cultures (Fig. 3) (*17, 18*), and it was nontoxic in the absence of exogenous glutamate (Fig. 3) (*12*). gp46 and p24 did not induce iNOS and were not toxic to rodent cultures (Fig. 3). Short 10– to 15–amino acid peptides in the proposed active region of gp41 also induced iNOS and caused neurotoxicity (*31*), confirming the specificity of gp41's toxic actions.

In summary, in severe AIDS dementia, iNOS appears to be coincidentally elevated with the increased expression of gp41. Our observations that human iNOS mRNA and protein levels parallel the expression of gp41 is consistent with other studies indicating that HIV-1–infected monocytes, as well as the viral coat proteins gp160 and gp41, induce expression of human iNOS (*17, 18*). Furthermore, monkeys infected with SIV who develop neurologic disease also have elevations of iNOS protein and catalytic activity that correlate with the levels of gp41 (*32*). The ability of NOS inhibitors to provide substantial protection against gp41-induced neurotoxicity implicates induction of iNOS and NO formation in neuronal killing. Because iNOS is elevated in other neurologic disorders, including multiple sclerosis, encephalitis, and sepsis, iNOS elevation may be a common end-pathway for many CNS inflammatory diseases (*33*).

The elevation of gp41 in the setting of severe (MSK 3 or 4) HIV-1 dementia suggests that the association among gp41 expression, severe dementia, and iNOS levels may be a

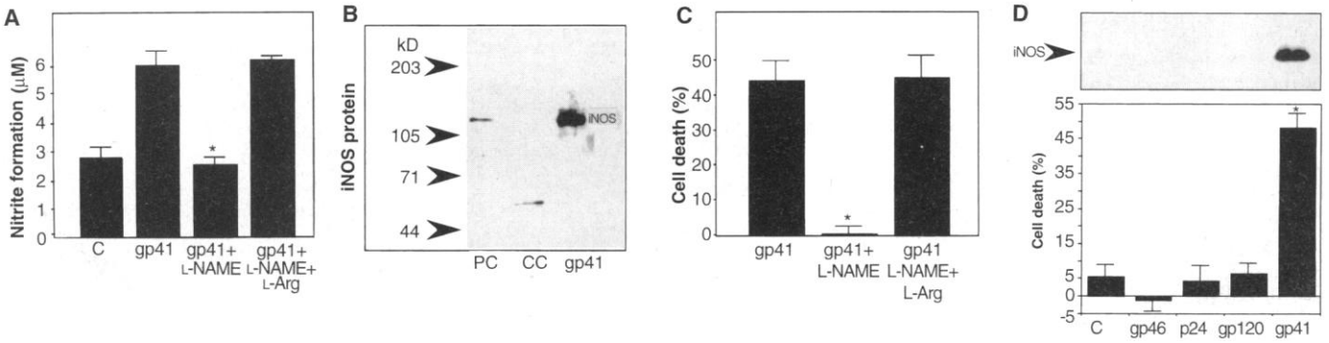

Fig. 3. gp41 induces NO-dependent neurotoxicity. (**A**) gp41 (100 nM) induced nitrite formation in a dose-dependent manner. Nitrite formation could be inhibited by the addition of 500 μM L-NAME, a competitive NOS inhibitor (*30*), and inhibition was reversed by excess 5 mM L-Arg. Data are means ± SEM for $n \geq 8$ from at least two experiments. Significance was determined by chi-square analysis with Student's *t* test for independent means for specific comparisons ($P \leq 0.01$ when gp41 was compared with control, and $P \leq 0.01$ when gp41 was compared with gp41 + 500 μM L-NAME). (**B**) gp41 induced iNOS protein in mixed neuronal cultures. Protein immunoblot analysis revealed the induction of iNOS protein by exposure to 100 nM gp41 for 7 days. These results were replicated three times with similar results (*30*). PC, positive control; CC, control cultures. (**C**) gp41 (100 nM) induced neurotoxicity in a dose-dependent manner. This neurotoxicity could be inhibited by the addition of 500 μM L-NAME, and protection was reversed by excess 5 mM L-Arg. Data are means ± SEM for $n \geq 8$ from at least two experiments. Toxicity was determined by exclusion of trypan blue, with 4000 to 8000 cells counted per data point (*30*). Significance was determined by chi-square analysis with Student's *t* test for independent means for specific comparisons ($P \leq 0.01$ when gp41 was compared with control, and $P \leq 0.01$ when gp41 was compared with gp41 + 500 μM L-NAME). (**D**) gp41 (100 nM) induced iNOS protein in mixed neuronal cultures and was neurotoxic, whereas other glycoproteins applied under identical conditions [HTLV-1 gp46 (100 nM), HIV-1 p24 (100 nM), and HIV-1 gp120 (100 nM)] failed to induce iNOS and were nontoxic. Protein immunoblot analysis revealed induction of iNOS protein by gp41 (100 nM) exposed for 7 days and accompanying neurotoxicity. Data are means ± SEM for $n \geq 8$ from at least two experiments. Toxicity was determined by exclusion of trypan blue, with 4000 to 8000 cells counted per data point (*30*) for $n \geq 8$ from two experiments. Significance was determined by chi-square analysis with Student's *t* test for independent means for specific comparisons [$P \leq 0.01$ when gp41 was compared with control (C), gp46, p24, and gp120].

reflection of cumulative viral burden in the brains of severely demented HIV-1 patients. However, an occasional HIV-1–infected patient may have high levels of viral protein in the CNS without dementia; and conversely, occasional patients with severe neurologic disease may have low levels of viral replication (*2*, *10*). Thus, other factors such as HIV-1 sequence heterogeneity may be important in the pathogenesis of HIV-1 dementia (*34*). Our previous studies have suggested that the severity of dementia correlates better with the degree of macrophage activation than with levels of HIV protein in the brain (*2*) or of HIV DNA (*10*). This suggests that productive CNS infection is necessary but not sufficient for the induction of neurological disease and is consistent with our observations that severe HIV dementia is significantly associated with elevated iNOS and gp41 levels. Because only severe dementia was associated with high levels of iNOS and gp41, we propose that there is a threshold effect in which a critical level of gp41 leads to iNOS induction and subsequent severe dementia. Why gp41 is elevated in severe HIV-1 dementia while p24 levels remain constant is not known. However, it is conceivable that gp41 may be more stable than other HIV-1 proteins because of its hydrophobic nature and retention in cellular membranes. Because gp41 is retained in cellular membranes, it may elicit a sustained immunologic reaction that triggers pathogenic processes ultimately leading to neuronal damage and dementia. In a manner analogous to that of nonsecreted membrane proteins that induce signaling processes through cell-to-cell interactions, we propose that gp41 expressed on infected cells interacts with adjacent cells to induce iNOS. Our observations would suggest that a high viral load, maybe even transiently, would increase the risk of development of dementia and the likelihood of more severe neurologic dysfunction. gp41 may be a key factor in mediating this effect; however, we cannot rule out the possibility that other viral proteins are contributing to the development of HIV-1 dementia. The potential linkage of iNOS induction to HIV-1–associated cognitive dysfunction implies that inhibitors of iNOS could exert therapeutic effects.

REFERENCES AND NOTES

1. B. Navia, B. Jordan, R. Price, *Ann. Neurol.* **19**, 517 (1986); R. Price *et al.*, *Science* **239**, 586 (1988); M. J. G. Harrison and J. C. McArthur, in *AIDS in Neurology* (Churchill-Livingston, New York, 1995), pp. 32–36.
2. J. D. Glass, H. Fedor, S. L. Wesselingh, J. C. McArthur, *Ann. Neurol.* **38**, 755 (1995).
3. S. Koenig *et al.*, *Science* **233**, 1089 (1986); D. W. Dickson and S. C. Lee, *J. Neuro-AIDS* **1**, 57 (1996); K. Takahashi *et al.*, *Ann. Neurol.* **39**, 705 (1996).
4. T. Saito *et al.*, *Neurology* **44**, 474 (1994); C. Tornatore, R. Chandra, J. R. Berger, E. O. Major, *ibid.*, p. 481.
5. I. P. Everall, P. J. Luthert, P. L. Lantos, *Lancet* **337**, 1119 (1991); C. A. Wiley *et al.*, *Ann. Neurol.* **29**, 651 (1991); E. Masliah *et al.*, *Lab. Invest.* **66**, 285 (1992).
6. B. A. Navia, E. Cho, R. W. Price, *Ann. Neurol.* **19**, 525 (1986); C. K. Petito and K. S. Cash, *ibid.* **32**, 658 (1992); C. Power *et al.*, *ibid.* **34**, 339 (1993).
7. J. D. Glass, S. L. Wesselingh, O. A. Selnes, J. C. McArthur, *Neurology* **43**, 2230 (1993).
8. R. Vazeux *et al.*, *Am. J. Pathol.* **140**, 137 (1992).
9. L. G. Epstein and H. E. Gendelman, *Ann. Neurol.* **33**, 429 (1993); S. A. Lipton and H. E. Gendelman, *N. Engl. J. Med.* **332**, 934 (1995).
10. C. A. Wiley and C. L. Achim, *Ann. Neurol.* **36**, 673 (1994); B. J. Brew, M. Rosenblum, K. Cronin, R. W. Price, *ibid.* **38**, 563 (1995); S. Pang *et al.*, *Nature* **343**, 85 (1990). Some studies suggest a correlation between the HIV-1 antigens in the brain and the presence of dementia, whereas other investigators do not find such a clear relation. Furthermore, HIV-1 antigen burden may not indicate viral burden [R. T. Johnson, J. D. Glass, J. C. McArthur, B. W. Chesebro, *Ann. Neurol.* **39**, 392 (1996)].
11. D. E. Brenneman *et al.*, *Nature* **335**, 639 (1988); S. A. Lipton, N. J. Sucher, E. B. Dreyer, *Neuron* **7**, 111 (1991); S. A. Lipton, *Trends Neurosci.* **15**, 75 (1992); T. Savio and G. Levi, *J. Neurosci. Res.* **34**, 265 (1993); E. B. Dreyer, P. K. Kaiser, J. T. Offermann, S. A. Lipton, *Science* **248**, 364 (1990).
12. V. L. Dawson, T. M. Dawson, G. R. Uhl, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3256 (1993).
13. E. Bonofoco *et al.*, *ibid.* **92**, 7162 (1995).
14. D. C. Adamson, T. M. Dawson, M. C. Zink, J. E. Clements, V. L. Dawson, *Mol. Med.* **2**, 417 (1996).
15. C. K. Petito and B. Roberts, *Am. J. Pathol.* **146**, 1121 (1995); H. Adlebiassette *et al.*, *Neuropath. Appl. Neurobiol.* **21**, 218 (1995); H. A. Gelbard *et al.*, *ibid.*, p. 208.
16. L. Wahl *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 621 (1989); H. A. Gelbard *et al.*, *J. Virol.* **68**, 4628 (1994); D. Giulian, K. Vaca, C. A. Noonan, *Science* **250**, 1593 (1990).
17. J. E. Merrill *et al.*, *J. Virol.* **66**, 2217 (1992); P. Koka *et al.*, *J. Exp. Med.* **182**, 941 (1995).
18. P. Genis *et al.*, *J. Exp. Med.* **176**, 1703 (1992); M. I. Bukrinsky *et al.*, *ibid.* **181**, 735 (1995); D. Pietraforte, E. Tritarelli, U. Testa, M. Minetti, *J. Leukocyte Biol.* **55**, 175 (1994); V. Mollace *et al.*, *Biophys. Biochem. Res. Commun.* **134**, 439 (1993).
19. C. C. Chao, S. Hu, T. W. Molitor, E. G. Shaskar, P. K. Peterson, *J. Immunol.* **149**, 2736 (1992); K. M. Boje and P. K. Arora, *Brain Res.* **587**, 250 (1992); V. L. Dawson, H. P. Brahmbhatt, J. A. Mong, T. M. Dawson, *Neuropharmacology* **33**, 1425 (1994); S. J. Hewett, C. A. Csernasnsky, D. W. Choi, *Neuron* **13**, 487 (1994).
20. J. E. Merrill, L. J. Ignarro, M. P. Sherman, J. Melinek, T. Lane, *J. Immunol.* **151**, 2132 (1993); B. Mitrovic, L. J. Ignarro, S. Montestruque, A. Smoll, J. E. Merrill, *Neuroscience* **61**, 575 (1994); A. J. Mackenzie-Graham, B. Mitrovic, A. Smoll, J. E. Merrill, *Dev. Neurosci.* **16**, 162 (1994); S. L. Wesselingh *et al.*, *Ann. Neurol.* **33**, 576 (1993); W. R. Tyor *et al.*, *ibid.* **31**, 349 (1992); W. R. Tyor *et al.*, *Neurology* **43**, 1002 (1993); D. E. Griffin, S. L. Wesselingh, J. C. McArthur *et al.*, *Ann. Neurol.* **35**, 592 (1994).
21. Tissue was collected at autopsy from a prospectively characterized population of AIDS patients, rapidly frozen in isopentane, and stored at –70°C. In 28 cases, cortical specimens were obtained from the frontal lobe. In one seronegative control patient, cortical tissue was derived from the cingulate gyrus. In three HIV-1–infected individuals, tissue samples from both frontal and parietal cortical regions were simultaneously assessed. One of these patients was nondemented, and two patients had mild and severe dementia, respectively. In all patients, the presence of CNS opportunistic infections or lymphoma was excluded by computed tomography or magnetic resonance imaging and cerebrospinal fluid analysis, as well as by postmortem histopathologic evaluation of brain tissue sections. HIV-1–seronegative control specimens were obtained from patients without CNS lesions. The causes of death in control patients included myocardial infarction, trauma, cirrhosis of the liver, atherosclerosis, and widespread cytomegalovirus infection. The mean ages and postmortem delays were similar for all patient groups, and CD4 counts did not differ between AIDS patients without dementia and those with mild or severe dementia. Postmortem delays in tissue collection were as follows: seronegative control, 15.0 ± 3.0 hours; HIV-1 positive (AIDS) with no dementia, 13.7 ± 1.8 hours; HIV-1 positive with mild dementia, 17.9 ± 3.3 hours; and HIV-1 positive with severe dementia 14.9 ± 3.1 hours. A mean CD4 count (number of cells per cubic millimeter) was not done for the seronegative control. It was 51.4 ± 29.1 for HIV-1–positive patients with no dementia; 38.6 ± 15.3 for HIV-1–positive patients with mild dementia; and 39.0 ± 24.0 for HIV-1–positive patients with severe dementia.
22. R. W. Price and B. J. Brew, *J. Infect. Dis.* **158**, 1079 (1988); R. S. Janssen *et al.*, *Neurology* **41**, 778 (1991). The patients were grouped according to MSK criteria for HIV-associated dementia as nondemented (MSK 0), mildly demented (MSK 1 or 2), or severely demented (MSK 3 or 4).
23. In previous experiments, we compared the expression of human iNOS mRNA to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA [L. Bö *et al.*, *Ann. Neurol.* **36**, 778 (1994)]. However, subsequent studies have shown that GAPDH mRNA and protein levels in the brain are regulated by NO [D. S. Bredt and S. H. Snyder, *Annu. Rev. Biochem.* **63**, 175 (1994)]. Thus, detection of β-actin mRNA was used to normalize the expression of iNOS mRNA in the phosphorimaging analysis of the PCR products. Reverse transcription of RNA and PCR amplification of iNOS and β-actin cDNA were carried out in a single PCR reaction to minimize variation in both RNA yield and amplification efficiency of the two transcripts. Total cellular RNA was extracted from frozen brain tissue with RNAzol B (Biotec, Houston, TX) according to the manufacturer's instructions. RNA was dissolved in 50 ml of diethyl pyrocarbonate–treated water and incubated for 10 min at 37°C and for 5 min at 95°C with 10 units of ribonuclease-free deoxyribonuclease I (Boehringer Mannheim) to remove chromosomal DNA contamination. Human iNOS and human β-actin transcripts were amplified in a single reaction tube with the use of the GeneAmp RNA PCR Kit (Perkin-Elmer–Cetus, Norwalk, CT). Complementary DNA was synthesized with 2 μg of total RNA and primed with random hexamers. The PCR reaction was carried out in the presence of 100 mM each of deoxynucleotide and the Taq Start antibody (Clontech, Palo Alto, CA). All other components were used as suggested by the manufacturer. Primers were designed to amplify a 237–base pair (bp) product of human iNOS and a 577-bp product of human β-actin. Amplimers used for amplification of human iNOS sequences were AAGCCCCAAGACCCAGTGCC (20-nucleotide oligomer, sense strand) and CCAGCATCTCCTCCTGGTAGAT (22-nucleotide oligomer, antisense strand). Human β-actin–specific amplimers were ATCTGGCACCACACTTCTACA (22-nucleotide oligomer, sense strand) and GTTTCGTGGATGCCACAGGACT (22-nucleotide oligomer, antisense strand). Each primer was added at 0.15 mM per reaction. PCR was performed in an OmniGene DNA thermal cycler (Labnet, Woodbridge, NJ). For detection of gene expression, samples were subjected to two phases of thermal cycling. After an initial denaturation step at 95°C for 2 min, PCR proceeded with five cycles at 95°C for 60 s, 60°C for 45 s, and 72°C for 90 s. Samples were then subjected to 40 cycles at 94°C for 45 s, 65°C for 45 s, and 72°C for 90 s. This was followed by an extension step at 72°C for 7 min. Because we expected a higher level of gene expression for β-actin as compared with that of iNOS, the addition of human β-actin–specific amplimers to the PCR reaction was delayed by 10 cycles. Negative controls were included at each RT-PCR reaction. PCR-amplified sequences (15 μl) were separated by electrophoresis through a 1.5% agarose gel, transferred to positively charged Hybond nylon membranes (Amersham), and cross-linked by ultraviolet irradiation. Oligonucleotide probes with specificity for the human iNOS and human β-actin PCR products were radiolabeled with [^{32}P]dATP (6000 Ci/mmol) by terminal transferase with the use of the DNA 3′ end-labeling kit (Boehringer Mannheim). The nucleotide sequences of the internal oligonucleotides were AGTTTC-

TGGCAGCAACGGCTCCATGACTCCCAGCACAG (40-nucleotide oligomer, sense strand) for iNOS and TGAGACCTTCAACACCCCAGCCATGTACGTTG-CTATCCAG (40-nucleotide oligomer, sense strand) for β-actin. Filters were prehybridized and hybridized in Church and Gilbert hybridization solution [G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984)]. Hybridization was performed at 60°C for 12 to 14 hours. The membranes were washed twice in 2× saline sodium citrate (SSC) and 0.1% SDS at room temperature for 10 min. This was followed by high-stringency washes in 0.2× SSC and 0.1% SDS at 60°C for 15 min. The filters were exposed to phosphor screens for 2 to 10 hours. The counts per band were measured by phosphorimaging (Molecular Dynamics) and used to determine the relative ratios of the intensity of the iNOS band to that of the β-actin band.

24. B. Wildemann, M. Sasaki, V. I. Christov, T. M. Dawson, V. L. Dawson, unpublished observations.
25. The iNOS PCR products obtained by several reactions were assessed by nucleotide sequence analysis. Sequencing was performed by the Genetics Resources CORE Facility of the Johns Hopkins University School of Medicine, with the use of the fluorescent dideoxy chain termination method using an automated DNA sequencer (model 373A, Applied Biosystems, Foster City, CA).
26. D. A. Geller *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3491 (1993).
27. Immunoblot analysis of iNOS, p24, gp41, gp120, and β-tubulin was performed as described [A. H. Sharp *et al.*, *Neuron* **14**, 1065 (1995)]. Briefly, tissues were homogenized in ice-cold 50 mM Hepes (pH 7.4) with 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, leupeptin (10 mg/ml), pepstatin A (10 mg/ml), aprotinin (1 mg/ml), and 1 mM EDTA, and centrifuged at 100,000*g* for 60 min. Polyacrylamide gel electrophoresis (PAGE) on a 5 to 16% gradient was used to separate proteins. After electrophoresis, proteins were electroblotted onto nitrocellulose and incubated with antibody to macNOS (1:500; Transduction Laboratories, Lexington, KY); antibody to p24 (HIV-1) (1:1000; Intracel, Cambridge, MA); antibody to gp41 (1:250; Intracel); antibody to gp120 (HIV-1), which recognizes recombinant gp120 and native gp120 from extracts of HIV-1–infected cells in protein immunoblots (1:250, Intracel); and antibody to β-tubulin (1:10000; Sigma), respectively. For gp41 protein analysis, equivalent amounts of protein lysate prepared from each of the pellet fractions after a 60-min 100,000*g* spin were resolved with PAGE on a 4 to 20% gradient. SDS (0.1%) was added to the tris-glycine buffer and Tween 20 (0.1%) was added to the phosphate-buffered saline for rinsing steps. Immunoblots were developed by enhanced chemoluminescence (Kirkegaard & Perry, Gaithersburg, MD). The specificity of the antibody to gp41 was demonstrated by immunoblots assessing the entire molecular weight range of 14 to 211 kD. gp41 revealed a single band at 41 kD only in HIV-1–infected tissue. Bands were scanned (Molecular Dynamics), and relative ratios of the intensity of the bands to that of the β-tubulin band were calculated.
28. S. M. Toggas *et al.*, *Nature* **367**, 188 (1994); J. M. Hill *et al.*, *Brain Res.* **603**, 222 (1993).
29. D. C. Adamson, J. D. Glass, J. C. McArthur, T. M. Dawson, V. L. Dawson, unpublished observations.
30. Recombinant $gp41_{IIIB}$ (amino acids 1 through 241, Intracel), recombinant p24 (amino acids 1 through 189; Intracel), recombinant $gp120_{IIIB}$ (amino acids 1 through 516; Intracel), and recombinant $gp46_{HTLV-1}$ (amino acids 26 through 215; Intracel) were used to investigate the potential induction of iNOS and neurotoxicity in primary mixed neuronal-glial cultures. Previous studies indicate that nonglycosylated recombinant gp41, as well as soluble peptides of gp41, are capable of inducing cytokines in both human and rodent cultures in a manner similar to that of glycosylated gp41 [P. Koka *et al.*, *J. Neuroimmunol.* 57, 179 (1995); P. Koka *et al.*, *J. Exp. Med.* **182**, 941 (1995)]. Primary cortical cell cultures were prepared from fetal rats at gestational day 16 in a procedure modified from that previously described [V. L. Dawson, H. P. Brahmbhatt, J. A. Mong, T. M. Dawson, *Neuropharmacology* **33**, 1425 (1994)]. Briefly, the cortex was dissected and the cells were dissociated by trituration in modified Eagle's medium (MEM), 20% horse serum, 25 mM glucose, and 2 mM L-glutamine after a 30-min digestion in 0.027% trypsin in saline solution. The cells were plated on 15-mm multiwell plates coated with polyornithine. Four days after plating, the culture medium was changed to MEM, 5% horse serum, 25 mM glucose, and 2 mM L-glutamine without phenol red. At this time, each protein was added in the presence or absence of L-NAME or L-Arg. The cultures were maintained in an 8% CO_2, humidified, 37°C incubator. The growth medium was supplemented with 10 μl of 2M glucose twice per week during the course of the experiment. Toxicity was assayed 7 days after initiation of exposure to the appropriate protein by trypan blue exclusion (0.4% trypan blue in CSS) as described (*12*). At least two separate experiments with four separate wells were performed, with a minimum of 4000 to 12,000 neurons counted per data point. The data were collected by an observer blinded to the treatment protocol. After 7 days, 400 μl of media was removed for colorimetric analysis of nitrite formation and added to 400 μl of Greiss reagent containing 4.25% phosphoric acid, 9.7 μM D-naphthyl ethylenediamine, and 0.14 mM sulfanilic acid. The samples were vortexed and after 5 to 10 min were read on a spectrophotometer at an absorbance of 563. The sample concentration was determined against a nitrite standard curve. For immunoblot analysis of iNOS, equivalent amounts of cell lysate prepared from the culture cells were loaded on a 5% SDS-PAGE gel (Bio-Rad, Hercules, CA) in tris-glycine buffer under reducing conditions and were further assessed as described for iNOS and β-tubulin assessment in cortical brain tissue.
31. D. C. Adamson, T. M. Dawson, V. L. Dawson, unpublished observations.
32. D. C. Adamson, C. M. Zink, J. E. Clements, T. M. Dawson, V. L. Dawson, unpublished observations; T. E. Lane, M. J. Buchmeier, B. B. Watry, H. S. Fox, *Mol. Med.* **2**, 27 (1996).
33. L. Bo *et al.*, *Ann. Neurol.* **36**, 778 (1994); M.-L. Wong *et al.*, *Nature Med.* **2**, 581 (1996).
34. C. Powers *et al.*, *J. Virol.* **68**, 4643 (1994). Demented and nondemented patients with AIDS differ in brain-derived HIV-1 envelope sequences.
35. The authors thank S. H. Snyder and R. T. Johnson for insightful critiques and discussion, A. Schmidt for secretarial assistance, and T. Billiar for the human iNOS cDNA. Supported in part by the following grants from the USPHS: NS07392 (D.C.A.), NS22643 (J.D.G., J.C.M., V.L.D., and T.M.D.), AI35042 (J.C.M.), RR007222 (J.C.M.), and Clinical Investigator Development Awards NS01577 (J.D.G.) and NS01578 (T.M.D.). V.L.D. is also supported by the American Foundation for AIDS Research; J.C.M. by the Charles A. Dana Foundation; and T.M.D. by the American Health Assistance Foundation, International Life Sciences Institute, and a Paul Beeson Physicians Scholars in Aging Research Award.

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Reduction of Voltage-Dependent Mg^{2+} Blockade of NMDA Current in Mechanically Injured Neurons

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Activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors is implicated in the pathophysiology of traumatic brain injury. Here, the effects of mechanical injury on the voltage-dependent magnesium (Mg^{2+}) block of NMDA currents in cultured rat cortical neurons were examined. Stretch-induced injury was found to reduce the Mg^{2+} blockade, resulting in significantly larger ionic currents and increases in intracellular free calcium (Ca^{2+}) concentration after NMDA stimulation of injured neurons. The Mg^{2+} blockade was partially restored by increased extracellular Mg^{2+} concentration or by pretreatment with the protein kinase C inhibitor calphostin C. These findings could account for the secondary pathological changes associated with traumatic brain injury.

Evidence from animals and humans has shown that the extracellular level of the excitatory neurotransmitter glutamate is elevated after traumatic brain injury (*1*, *2*). Elevated glutamate contributes to delayed tissue damage, presumably through activation of Ca^{2+}-permeable NMDA receptor channels. Treatment with NMDA receptor-channel antagonists has been reported to limit neurological dysfunction and partially preserve the bioenergetic state of posttraumatic brain tissue (*1*, *3*). In vitro, delayed neurodegeneration produced by using a plastic stylet to mechanically injure neocortical neurons in culture is attenuated by the NMDA antagonists dextrorphan or D-2-amino-5-phosphonovalerate (*4*). Despite the suggested involvement of NMDA receptors in these secondary pathological changes, it is not known whether mechanical injury directly affects NMDA channel properties in neurons of the central nervous system. Furthermore, because Mg^{2+} deficiency exacerbates, and increased extracellular Mg^{2+} attenuates, the pathological outcome of posttraumatic brain tissue (*5*), we hypothesized that voltage-dependent Mg^{2+} block of NMDA receptor channels (*6*) might be altered in injured neurons.

To examine the effects of mechanical stretch injury on NMDA currents, we used the whole-cell patch clamp technique (*7*)

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