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Herpes simplex virus type 1 infection induces oxidative stress and the release of bioactive lipid peroxidation by-products in mouse P19N neural cell cultures

Jerry H Kavouras,[1] Emese Prandovszky,[1] Klara Valyi-Nagy,[1] S Krisztian Kovacs,[1] Vaibhav Tiwari,[2,3] Maria Kovacs,[2] Deepak Shukla,[2,3] and Tibor Valyi-Nagy[1]

Departments of [1]Pathology, [2]Ophthalmology and Visual Sciences, and [3]Microbiology and Immunology, University of Illinois at Chicago, College of Medicine, Chicago, Illinois, USA

To determine whether herpes simplex virus type 1 (HSV-1) infection causes oxidative stress and lipid peroxidation in cultured neural cells, mouse P19 embryonal carcinoma cells were differentiated into cells with neural phenotypes (P19N cells) by retinoic acid and were then infected with HSV-1. Cellular levels of reactive oxygen species (ROS) and the release of lipid peroxidation by-products into the tissue culture medium were then measured by the generation of fluorescent markers hydroxyphenyl fluorescein and a stable chromophore produced by lipid peroxidation products, malondialdehyde (MDA) and hydroxyalkenals (4-HAEs; predominantly 4-hydroxy-2-nonenal [HNE]), respectively. HSV-1 infection increased ROS levels in neural cells as early as 1 h post infection (p.i.) and ROS levels remained elevated at 24 h p.i. This viral effect required viral entry and replication as heat- and ultraviolet light–inactivated HSV-1 were ineffective. HSV-1 infection also was associated with increased levels of MDA/HAE in the culture medium at 2 and 4 h p.i., but MDA/HAE levels were not different from those detected in mock infected control cultures at 1, 6, and 24 h p.i. HSV-1 replication in P19N cells was inhibited by the antioxidant compound ebselen and high concentrations of HNE added to the cultures, but was increased by low concentrations of HNE. These findings indicate that HSV-1 infection of neural cells causes oxidative stress that is required for efficient viral replication. Furthermore, these observations raise the possibility that soluble, bioactive lipid peroxidation by-products generated in infected neural cells may be important regulators of HSV-1 pathogenesis in the nervous system. *Journal of NeuroVirology* (2007) **13,** 416–425.

Keywords: 4-hydroxy-2-nonenal; HSV-1; lipid peroxidation; malondialdehyde oxidative stress; neural cell culture

Introduction

Herpes simplex virus type 1 (HSV-1) is the primary causative agent of sporadic encephalitis (reviewed by Skoldenberg, 1996; Whitley, 2001). During this life-threatening disease, there is viral replication in neurons and non-neuronal cells, infiltration of the brain by acute and chronic inflammatory cells, and tissue necrosis and apoptosis of neuronal and non-neuronal cells (Skoldenberg, 1996; Johnson and Valyi-Nagy,

Address correspondence to Tibor Valyi-Nagy, MD, PhD, University of Illinois at Chicago, College of Medicine, Department of Pathology, Room 130, 840 South Wood Street, M/C 847, Chicago, IL 60612, USA. E-mail: tiborv@uic.edu

The current address of Jerry H. Kavouras is Department of Biology, Lewis University, Romeoville, Illinois, USA.

Jerry H. Kavouras and Emese Prandovszky contributed equally to this work.

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1998; Aurelian, 2005). Studies using human autopsy tissues and experimental HSV-1 infection of laboratory animals suggest that damage to neural tissues including neuronal apoptosis is not limited to infected cells (Valyi-Nagy *et al*, 2000; Shaw *et al*, 2002; Milatovic *et al*, 2002; Aurelian, 2005). Survivors suffer severe neurological sequelae due to permanent damage to neural structures including extensive loss of neurons.

The mechanisms of brain injury during HSV-1 encephalitis are not well understood. Previous observations by our laboratory have indicated that one mechanism by which HSV-1 infection causes brain injury is oxidative damage (Valyi-Nagy *et al*, 2000; Milatovic *et al*, 2002; Valyi-Nagy and Dermody, 2005). Specifically, HSV-1 encephalitis in mice is associated with nucleic acid damage due to hydroxyl radical attack, lipid peroxidation, and protein damage mediated by the lipid peroxidation by-product 4-hydroxy-2-nonenal (HNE) (Valyi-Nagy *et al*, 2000; Milatovic *et al*, 2002; Valyi-Nagy and Dermody, 2005). In these studies, oxidative damage affected both neurons and non-neuronal cells and localized to brain regions that showed histopathologic and immunohistochemical evidence of HSV-1 infection and reactive inflammation (Valyi-Nagy *et al*, 2000; Milatovic *et al*, 2002; Valyi-Nagy and Dermody, 2005).

The molecular mechanisms by which HSV-1 infection causes oxidative brain damage are not well understood and the relative contributions of direct viral toxic effects and that of reactive inflammatory processes are unknown. It is, however, likely that the immune and inflammatory responses of the host play an important role in the pathogenesis of oxidative brain injury during HSV-1 encephalitis. Natural killer lymphocytes, macrophages/microglial cells, specific CD4+ and CD8+ T cells, specific antibody-producing B lymphocytes, and to a lesser extent polymorphonuclear phagocytes all participate in the immune/inflammatory response to HSV-1 infection of the nervous system (reviewed by Whitley, 2001; Ellermann-Eriksen, 2005). Acute HSV-1 infection is associated with locally increased levels of a number of cytokines including tumor necrosis factor α (TNFα) as well as an increased activity of the inducible form of nitric oxide synthetase (iNOS) (Koprowski *et al*, 1993; Shimeld *et al*, 1997; Meyding-Lamade *et al*, 1998; Fujii *et al*, 1999). Nitric oxide (NO) and TNFα have anti-HSV-1 activity and can either generate potent oxidizing radical byproducts through reactions directly, e.g., NO, or induce free radical–mediated injury indirectly through membrane signaling, e.g., TNFα (Matthews *et al*, 1987; Liochev an Fridovich, 1997; Sanchez-Alcazer *et al*, 2000). Superoxide radical (O_2^-) and other ROS may be generated by infiltrating NADPH oxidase–expressing phagocytic cells and by humoral responses involving xanthine oxidase (XO) in inflamed tissues (Akaike, 2001). NO and ROS, particularly O_2^-, may form reactive nitrogen oxides like peroxynitrite ($ONOO^-$) that are particularly potent oxidants of proteins, nucleic acids, and membrane unsaturated lipids. Importantly, free radicals can cause lipid peroxidation, an autocatalytic process that damages lipid-containing structures and yields soluble, highly reactive by-products, primarily 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA). HNE and MDA are electrophilic species that can covalently modify and damage cellular macromolecules. HNE induces cell death and DNA fragmentation in a dose-dependent manner (Kruman, 1997; Camandola, 2000; Soh *et al*, 2000; Kalyankrishna, 2002).

Whether stimuli related directly to HSV-1 infection of neural cells participate in the pathogenesis of oxidative brain injury during HSV-1 encephalitis is unknown. HSV-1 infection causes lipid peroxidation in cultured HeLa cells and induces NO production in differentiated monocytes and macrophages in culture (Palu *et al*, 1994; Lopez-Guerrero and Alonso, 1997; Fujioka *et al*, 2000). However, it is not known whether HSV-1 infection of neural cells (neurons or glial cells) can directly induce reactive oxygen species (ROS). Neither is it known whether HSV-1 infection of neural cells leads to lipid peroxidation and the release of soluble and highly bioactive lipid peroxidation products like HNE and MDA from infected cells.

To determine whether HSV-1 infection causes oxidative stress and lipid peroxidation in cultured neural cells, in the current study mouse P19 embryonal carcinoma cells were differentiated into cells with neural phenotypes by retinoic acid and were then infected with HSV-1. Neurally differentiated mouse P19 cells (P19N cells) similarly to neurally differentiated human embryonal carcinoma NT2 (NTera 2) cells have been widely used as experimental platforms to study neural cells (Jones-Villeneuve *et al*, 1982; Andrews, 1984; Tamagno *et al*, 2000). We report here that HSV-1 infection increases ROS levels in neural cells and is also associated with the transiently increased release of soluble lipid peroxidation by-products from P19N cultures during the early stages of the infection. Furthermore, HSV-1 replication in P19N cells is inhibited by the antioxidant compound ebselen suggesting that oxidative stress is required for efficient viral replication in neural cells. HSV-1 replication in P19N cells is also inhibited by high concentrations of HNE, but is increased by low concentrations of HNE. These findings suggest that HSV-1–induced oxidative stress plays an important role in the pathogenesis of HSV-1 encephalitis and that viral effects may directly contribute to oxidative tissue damage caused by HSV-1 encephalitis. Furthermore, these data suggest that HSV-1 infection–induced soluble, bioactive lipid peroxidation by-products may be mediators of HSV-1–induced tissue damage of infected and bystander cells as well as regulators of HSV-1 replication in the nervous system.

Results

Expression of HSV-1 proteins and HSV-1 replication in P19N neural cultures

To determine whether HSV-1 proteins are expressed in P19N neural cells, P19N cells grown on chamber slides were either inoculated with 1 plaque-forming unit (PFU)/cell of HSV-1 strain KOS or were mock infected with virus free Vero cell extract and the expression of HSV-1 proteins was determined by immunocytochemistry using a polyclonal anti-HSV-1 antibody at 24 h post infection (p.i.) (Figure 1). In HSV-1–infected cultures, nearly all cells were immunoreactive for HSV-1 proteins (Figure 1B). No staining was detected in mock-infected cultures (Figure 1A) and in virus-infected cultures exposed to secondary antibody only (data not shown). These observations indicate that HSV-1 proteins are expressed in P19N cells following HSV-1 inoculation.

To determine whether HSV-1 infects and replicates in P19N neural cultures, P19N cells were inoculated with HSV-1 strain KOS at a multiplicity of infection (MOI) of 1 PFU/cell, incubated at 37°C for 1 h with occasional gentle agitation, washed twice in sterile phosphate-buffered saline (PBS), and further incubated in fresh medium for 24 h. The amount of infectious HSV-1 in the cultures was determined by plaque assay using Vero cells at 1, 6, and 24 h p.i. Whereas at 1 and 6 h p.i. HSV-1 KOS levels in the cultures were significantly lower than the amount of input virus, at 24 h p.i. KOS titers increased to levels similar to that of input virus (Figure 2). These observations indicate that HSV-1 can replicate in P19N cells.

Increased generation of ROS in HSV-1–infected neural cultures

To determine whether HSV-1 infection induces ROS in neural cultures, P19N cells were inoculated with HSV-1 KOS at a MOI of 1 PFU/cell, or with Vero cell extract (mock infection) or with sterile PBS and fluorescence of hydroxyphenyl fluorescein (HPF) was determined at selected times after treatment. Nonfluorescent HPF becomes fluorescent in presence of reactive oxygen species, particularly hydroxyl radicals. HSV-1–inoculated cultures demonstrated significantly higher fluorescence than cell extract or PBS-treated cultures at 1, 2, 3, and 24 h p.i. (Figure 3). Specifically, HPF fluorescence values in KOS inoculated cultures were 21.5%, 69.8%, 45.7%, and 80% higher than in PBS-inoculated cultures at 1, 2, 3, and 24 h, respectively ($P < .01$ for all time points). Fluorescence values in KOS-inoculated cultures were 38.2%, 32.4%, 35.6%, and 54.3% higher than in Vero cell extract–inoculated (mock-infected) cultures at 1, 2, 3, and 24 h, respectively ($P < .01$ for all time points). Inoculation of P19N cultures with heat- or UV-inactivated HSV-1 did not induce significantly changed ROS levels (Figure 3). Significantly increased HPF fluorescence, however, was observed following inoculation of P19N cells with HSV-1 strain F and following inoculation of human neural (NT2N) cells with HSV-1 KOS (data not shown). These findings indicate that HSV-1 infection of neural cells is associated with increased generation of ROS and suggest that increased generation of ROS following HSV-1 infection of neural cells requires viral entry and replication.

HSV-1 infection causes lipid peroxidation in neural cultures

To determine whether HSV-1 infection causes lipid peroxidation in cultured neural cells, P19N cultures were either inoculated with HSV-1 (KOS, MOI = 1 PFU/cell), or were exposed to Vero cell extract (mock infection) or PBS. The combined level of lipid peroxidation products, malondialdehyde and

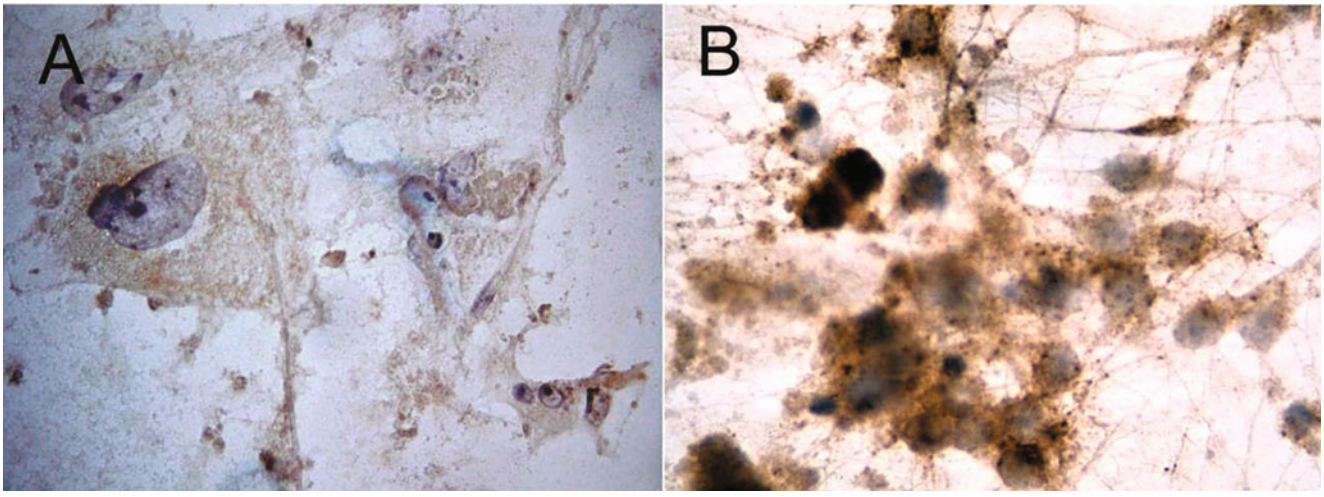

Figure 1 Expression of HSV-1 proteins in P19N neural cultures. P19N cells were either inoculated with 1 PFU/cell of HSV-1 strain KOS or were mock infected with virus free Vero cell extract and the expression of HSV-1 proteins was determined by immunocytochemistry using a polyclonal anti-HSV-1 antibody at 24 hours post infection (p.i.). **A,** Mock infection; **B,** HSV-1 infection. Brown color indicates positive staining.

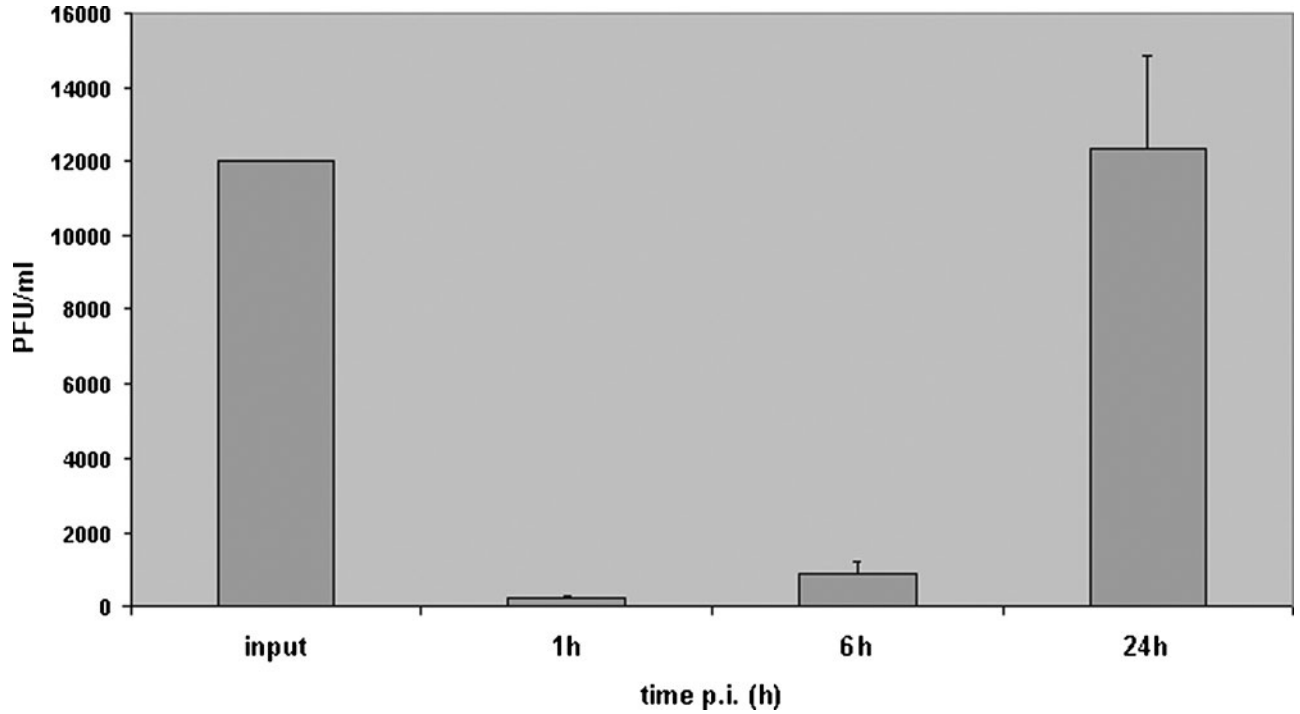

Figure 2 HSV-1 replication in P19N neural cultures following inoculation with 1 PFU/cell of HSV-1 strain KOS as determined by plaque assay. Standard deviation is provided with bars.

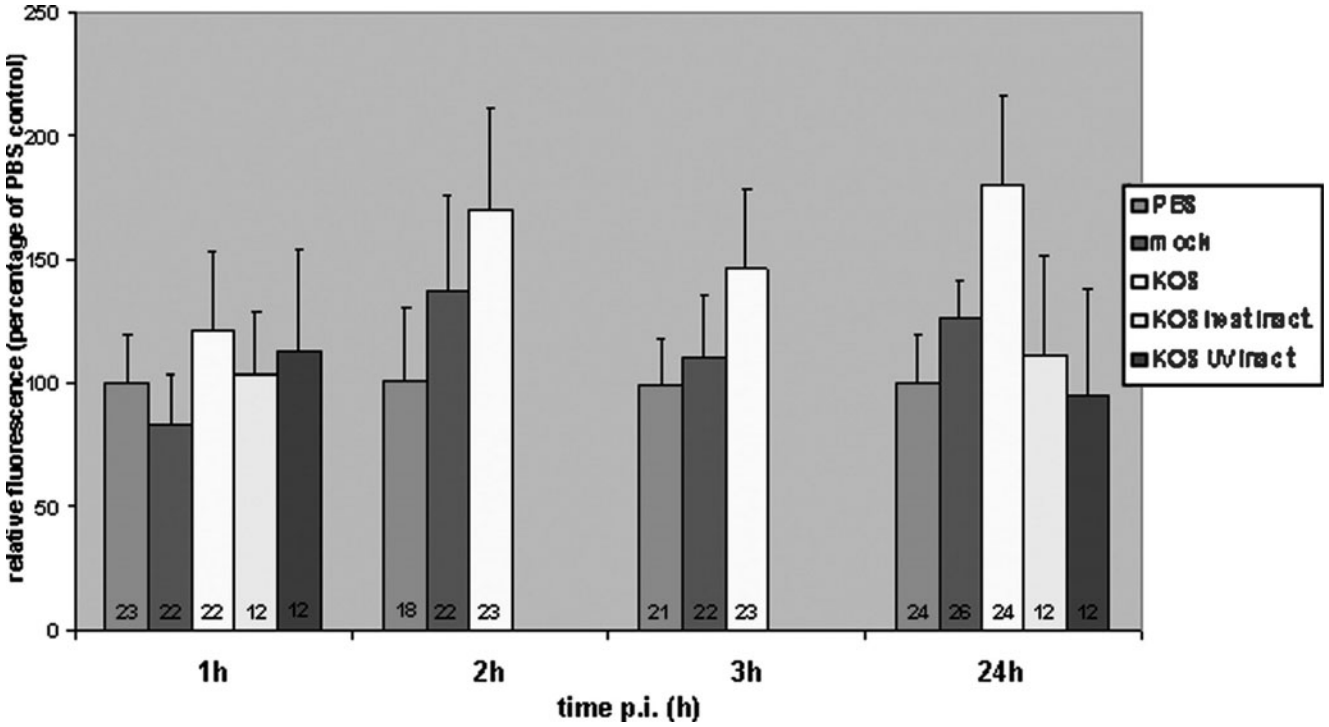

Figure 3 Generation of reactive oxygen radicals (ROS) in P19N and NT2N neural cells 1, 2, 3, and 24 h following HSV-1 inoculation (KOS, MOI = 1 PFU/cell), or exposure to PBS or Vero cell extract (mock infection) as detected by hydroxyphenyl fluorescein (HPF) fluorescence. Fluorescence measurements were made using a spectrofluorometer with excitation 490 nm and emission 515 nm and results are shown as percent change relative to PBS-treated control cultures. The number of examined cultures in each group is provided within columns and standard deviation is provided with bars. KOS heat inact. = heat inactivated KOS; KOS UV inact. = ultraviolet light-inactivated KOS.

hydroxyalkenals (4-HAE [predominantly HNE]), was then determined in the culture supernatants at 1, 2, 4, 6, and 24 h after HSV-1 infection, mock infection, or PBS treatment using the LPO-586 assay (Bioxytech LPO-586). HSV-1 infection was associated with increased levels of MDA/HAE in the culture medium at 2 and 4 h p.i., but MDA/HAE levels were not different from those detected in mock infected and PBS-treated control cultures at 1, 6, and 24 h p.i. (Figure 4). Specifically, at 2 h following KOS inoculation, MDA/HAE levels were at 358.00 (SD: 20.56) pmol/μg protein, significantly higher than MDA/HAE levels 2 h following PBS treatment (296.60 [SD: 2.96] pmol/μg protein; $P < .01$), or Vero cell extract inoculation (mock infection) (302.00 [SD: 10.10] pmol/μg protein; $P < .01$). At 4 h following KOS inoculation, MDA/HAE levels were at 486.00 (SD: 57.69) pmol/μg protein, significantly higher than MDA/HAE levels 4 h following PBS treatment (302.66 [SD: 9.20] pmol/μg protein; $P < .01$), or Vero cell extract inoculation (mock infection) (310.20 [SD: 8.49] pmol/μg protein; $P < .01$). These results indicate that HSV-1 infection of neural cells induces lipid peroxidation and the release of soluble MDA/4-HAE into the culture medium.

Effect of antioxidant and HNE treatment on HSV-1 replication in P19N cells

To determine whether antioxidant or HNE treatment affects HSV-1 replication in P19N neural cultures, P19N cells were inoculated with HSV-1 strain KOS at a MOI of 0.01 PFU/cell, incubated at 37°C for 1 h with occasional gentle agitation, washed twice in sterile PBS, and further incubated in fresh medium for 24 h. Some P19N cultures were treated with 5, 10, or 25 μM of the seleno-organic antioxidant compound ebselen, or with 5 or 50 μM of HNE for 1 h prior to and for 24 h following virus inoculation. The production of infectious HSV-1 in the cultures was determined by plaque assay 24 h p.i. (Figure 5). Ebselen treatment reduced HSV-1 yields in a dose dependent fashion. Relative to untreated KOS infected cultures (100% HSV-1 yield), virus yields were reduced to 38.75% (SD: 4.57%) by 5 μM of ebselen, to 35.25% (SD: 3.59%) by 10 μM of ebselen, and to 12.25% (SD: 2.06%) by 25 μM of ebselen ($P < .01$ for all three ebselen doses). Virus yields were also reduced by 50 μM HNE treatment to 56.0% (SD: 20.94%) ($P < .01$). In contrast, virus yields were increased to 165% (SD: 61.80%) by 5 μM of HNE ($p < 0.01$). To determine whether the ebselen and HNE concentrations used were toxic to P19N cells, P19N cells were exposed to 5, 10, or 25 μM of ebselen, or to 5 or 50 μM of HNE in the culture medium without concomitant HSV-1 inoculation and the cultures were observed under an inverted light microscope for 26 h. No cytotoxic effects were noted following treatment with 5, 10, or 25 μM of ebselen or with 5 μM of HNE, consistent with previous studies that also indicated that these doses correspond

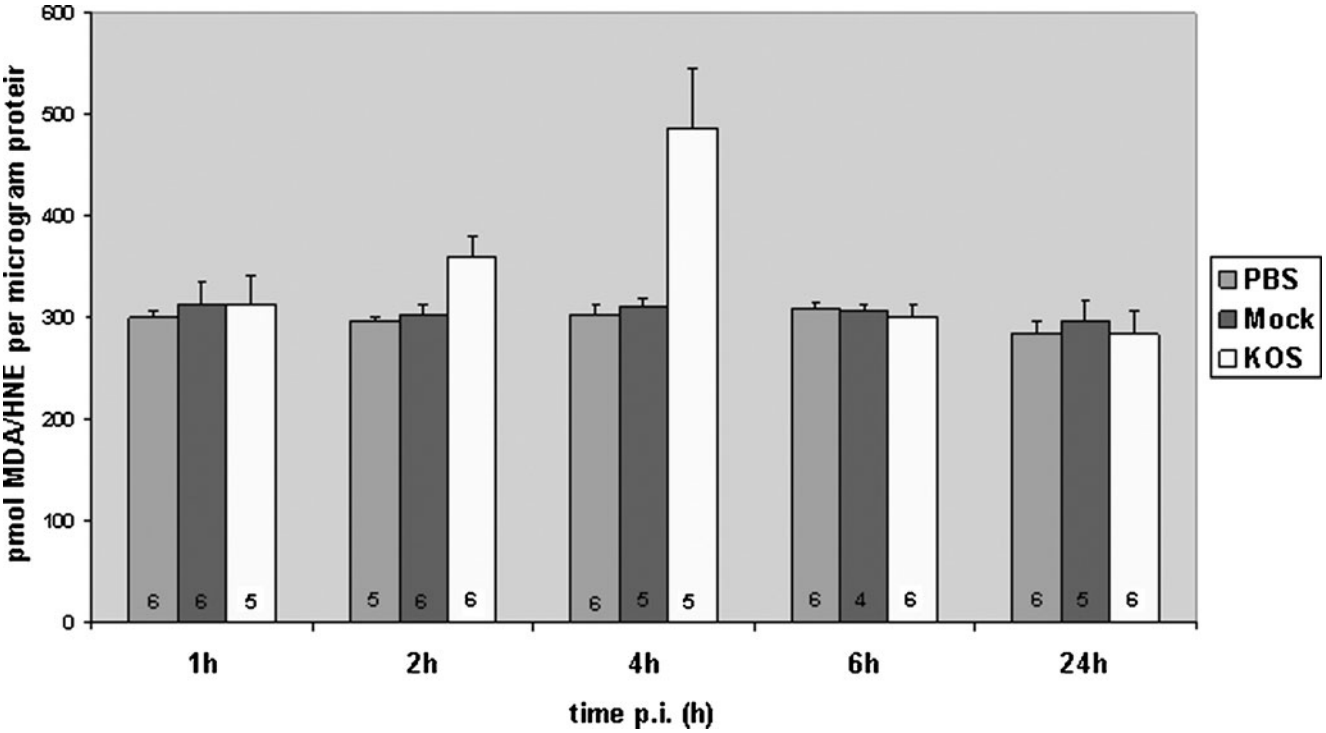

Figure 4 Concentrations of lipid peroxidation products malondialdehyde and hydroxyalkenals (4-HAE [predominantly HNE]) in the supernatant of P19N neural cells 1, 2, 4, 6, and 24 h following HSV-1 inoculation (strain KOS, MOI = 1 PFU/cell), or exposure to PBS or Vero cell extract (mock infection) as detected by LPO-586 assay (Bioxytech LPO-586). MDA was used as standard and results are expressed as pmol MDA equivalents/μg protein. Total protein in each sample was determined using Coomassie Plus Protein Assay Kit (Pierce). The number of examined cultures in each group is provided within columns and standard deviation is provided with bars.

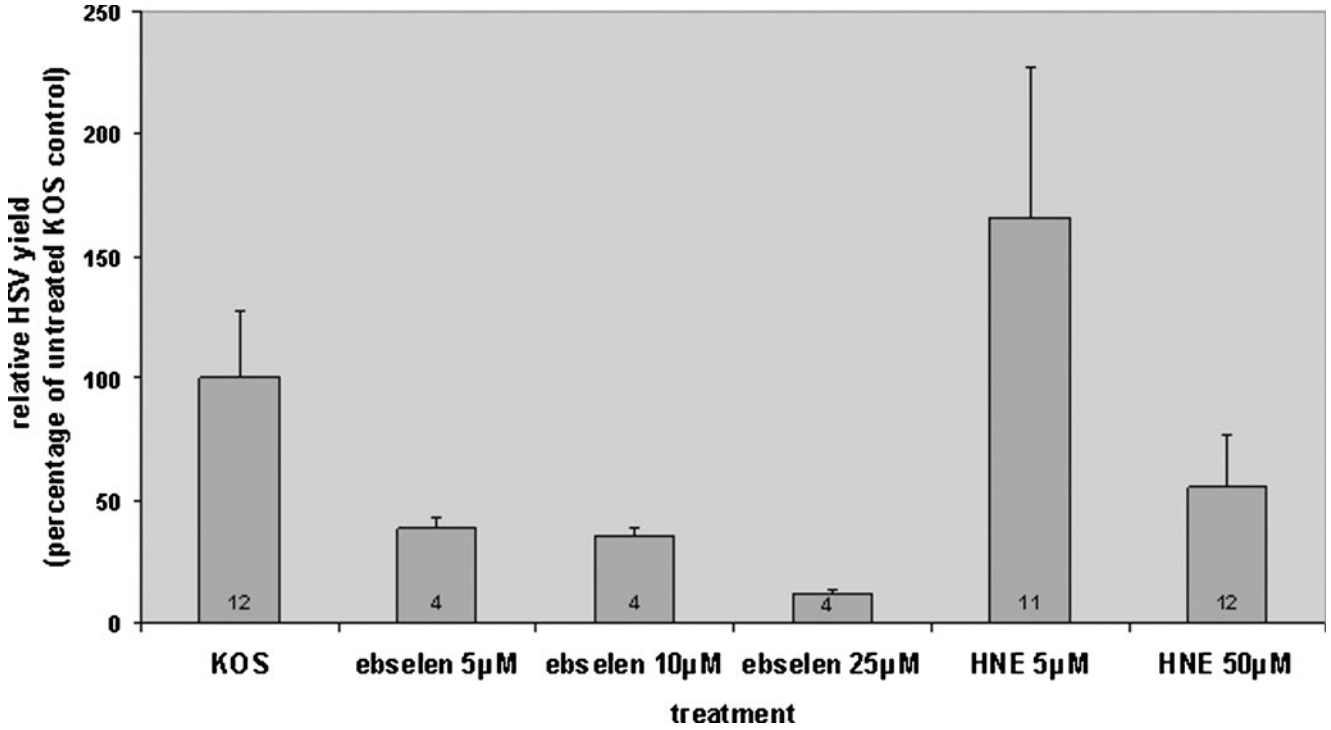

Figure 5 Effect of ebselen and HNE on HSV-1 replication in P19N neural cells as determined by plaque assay. P19N cells were inoculated with 0.01 PFU/cell of HSV-1 strain KOS and plaque assay was performed 24 h p.i. Cultures were treated with 5, 10, or 25 μM of ebselen, or with 5 or 50 μM of HNE for 1 h prior to and for 24 h following virus inoculation. The number of examined cultures at each experimental group is provided within columns and standard deviation is provided with bars.

to physiologic concentrations (Hardej and Trombetta, 2002; Montine *et al*, 1996). However, significant toxicity with approximately 50% cell loss was detected 1 day following treatment with 50 μM of HNE, a finding similar to previous reports (Montine *et al*, 1996).

These findings indicate that the production of infectious HSV-1 in P19N cells is significantly reduced by the antioxidant compound ebselen and high concentrations of HNE, but is increased by treatment with low concentrations of HNE. These findings also suggest that high concentrations of HNE may reduce HSV-1 replication in P19N cultures by reducing the number of viable cells.

Discussion

This study demonstrates that HSV-1 replicates in P19N neural cells and that HSV-1 infection leads to increased ROS levels in these cells (Figures 1, 2, and 3). We have found that HSV-1 infection leads to increased ROS levels as early as 1 h p.i. and ROS levels remain elevated in infected cells until 24 h p.i. We have also provided evidence suggesting that the increased generation of ROS following HSV-1 infection of neural cells requires viral entry and replication because infection with heat- or UV-inactivated HSV-1 did not induce increased ROS levels (Figure 3). We also have demonstrated that HSV-1 infection induces increased levels of soluble lipid peroxidation by-products MDA/HAE in the culture medium of neural cells at the early stages of the infection (Figure 4). Finally, we have found that HSV-1 replication in P19N cells is inhibited by the treatment of cultures with the antioxidant compound ebselen and high concentrations of HNE, but is increased by treatment with low concentrations of HNE.

The observation that HSV-1 infection leads to increased ROS levels in neural cells is novel and extends our existing knowledge related to the capacity of viruses to directly cause oxidative stress in cells. In tissue culture systems, a number of viruses have been found to induce increased production of ROS. Such effects of viral infection have been described in studies of differentiated monocytes and macrophages, murine neuroblastoma cells, and coronary artery smooth muscle cells following infection with HSV-1 (Lopez-Guerrero and Alonso, 1997; Fujioka *et al*, 2000), Japanese encephalitis virus (Liao *et al*, 2002, Lin *et al*, 2004), and human cytomegalovirus (CMV) (Speir, 2000), respectively. Other prominent examples involve the induction of iNOS expression in mixed neuronal-glial cultures by the human immunodeficiency virus (HIV) proteins gp41 and gp120 (Adamson *et al*, 1999; Walsch *et al*, 2004) and induction of ROS by gp120 in glial cultures (Viviani *et al*, 2001). The mechanisms of virus-induced ROS induction in many instances are not well understood, but most likely range from receptor-mediated

signaling induced by virion components to cytotoxic effects of viral proteins expressed during viral replication (Palu *et al*, 1994; Lin *et al*, 2004; Lopez-Guerrero and Alonso, 1997; Fujioka *et al*, 2000; Speir, 2000; Liao, 2002; Walsch *et al*, 2004). The mechanism(s) involved in HSV-1–induced oxidative stress in neural cells remains unknown at this point; however, our current study suggests that the increased generation of ROS following HSV-1 infection of neural cells requires viral entry and replication. The detection of increased ROS levels in neural cultures as early as 1 h following HSV-1 inoculation suggests that newly expressed viral immediate early or early gene products are sufficient for HSV-1–induced oxidative stress.

Our current study shows that HSV-1 replication in P19N cells is inhibited by the antioxidant compound ebselen, suggesting that HSV-1–induced oxidative stress is required for efficient viral replication in neural cells. These observations extend previous studies indicating that antioxidants can inhibit HSV-1 replication *in vitro* (Palamara *et al*, 1995; Savi *et al*, 2005). It is also noteworthy that antioxidant therapy has been reported to decrease the severity of HSV-1 ocular infection in mice (Nucci *et al*, 2000). The mechanism by which oxidative stress would promote HSV-1 replication in neural cells is not evident from our findings. However, oxidative stress can activate nuclear factor (NF)-κB and a variety of other cell activation signals that are known to participate in the regulation of the HSV-1 replication cycle (Akaike, 2001; Valyi-Nagy and Dermody, 2005; Roizman and Knipe, 2001). In fact, activation of NF-κB in HSV-infected macrophages has been reported to be dependent on oxidative stress (Mogensen *et al*, 2003).

ROS induced during viral infections may overcome antioxidant defenses to cause oxidative damage to host tissues and the infecting virus (reviewed by Akaike, 2001; Valyi-Nagy and Dermody, 2005). We have demonstrated here that HSV-1 infection induces increased levels of soluble lipid peroxidation by-products MDA/HAE (predominantly HNE) in the culture medium of neural cells at the early stages of the infection. This observation indicates that HSV-1 infection can directly cause oxidative damage in neural cells *in vitro* and suggest that direct HSV-1 effects may contribute to oxidative tissue damage caused by HSV-1 encephalitis. It is interesting that although HSV-1 induced persistently elevated ROS (oxidative stress) in neural cells in our studies (Figure 3), lipid peroxidation (oxidative damage) was observed only during the early stages of the infection (Figure 4). The mechanism and relevance of this observation is unclear at this point. However, it is possible that neural cells can mount an effective antioxidant defense during the later stages of HSV-1 infection. In that respect it is noteworthy that increased antioxidant enzyme activity following HSV-1 infection has been reported in non-neural tissue culture systems (Ray and Enquist, 2004). It should also be noted that the possibility of the expression of an HSV-1 gene(s) with antioxidant function at the later stages of the viral replication cycle in P19N cells cannot be excluded.

The demonstration that HSV-1 infection of neural cells is associated with an increased release of highly bioactive lipid peroxidation by-products MDA and HNE *in vitro* raises the possibility of a novel mechanism by which HSV-1 may affect the physiology and may damage uninfected bystander cells in the nervous system *in vivo*. At physiologic concentrations, HNE and MDA are potent regulators of cell growth and differentiation, affecting both cellular transcription and cell cycle progression (Zarkovic, Ilic *et al*, 1993; Keller and Mattson, 1998; Ji *et al*, 1998 and 2000; Kakashita and Hattori, 2001; Poli and Schaur, 2000; Semlitsch *et al*, 2002). In neuronal cell cultures, HNE has been reported to activate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, inhibit NF-κB activity, and induce apoptosis in a dose dependent manner (Kruman, 1997; Camandola, 2000; Soh *et al*, 2000; Kalyankrishna, 2002). NF-κB activity and other cell activation signals as well as the differentiation state of cells are well known to be affected by HSV-1 infection and are known to participate in the regulation of the HSV-1 replication cycle (reviewed by Roizman and Knipe, 2001). Thus, HNE and MDA released from HSV-1–infected cells may not only induce cell death in uninfected bystander cells but may also modulate their permissiveness to HSV-1 infection in the nervous system. Our findings reported here indicating that HNE can modulate HSV-1 replication in P19N cells provide strong support for this latter possibility.

In summary, observations reported here may lead to a better understanding of the pathogenesis of HSV-1 encephalitis, including the mechanisms of tissue injury and the regulation of viral replication in neural tissues. Future studies aimed at a better understanding of the pathogenesis and clinical significance of HSV-1-induced oxidative neural injury may help to identify novel therapeutic strategies against HSV-1 encephalitis.

Materials and methods

Cells

Mouse embryonal carcinoma P19 cells (ATCC, CRL-1825) were maintained at 37°C in a 5% CO_2 containing atmosphere using a growth medium consisting of 90% α-minimum essential medium (Gibco), 7.5% calf serum (Gibco), and 2.5% fetal bovine serum (Gibco) (Jones-Villeneuve *et al*, 1982). Penicillin-streptomycin (Gibco) was added to the growth medium, 50 U/ml and 50 μg/ml final concentration, respectively.

Human embryonal carcinoma NT2 (NTera 2) cells (ATCC, CRL-1973) were maintained at 37°C, in 5% CO_2 with a growth medium consisting of Dulbecco's

modified Eagle's medium with 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate and supplemented 10% fetal bovine serum (Gibco) (Andrews, 1984; Tamagno *et al*, 2000).

Virus

HSV-1 wild-type strain F was obtained from Dr. B. He (Department of Microbiology and Immunology, University of Illinois at Chicago). Virus stocks of HSV-1 wild type strain KOS (available in our laboratory) and of strain F were prepared and titered using Vero cells as previously described (Valyi-Nagy *et al*, 1991). Heat (65°C for 30 min) and ultraviolet (UV) light inactivation of viruses was performed as previously described (Valyi-Nagy *et al*, 1991). For mock infected controls, cells extracts were prepared from uninfected Vero cell cultures following the same protocol that was used for the preparation of virus stocks from HSV-1–infected Vero cell cultures.

Differentiation of P19 and NT2 cells into neural cell cultures (P19N, NT2N)

P19 and NT2 cells were differentiated by treatment with retinoic acid according to established protocols (Jones-Villeneuve *et al*, 1982; Andrews, 1984). P19 cells were grown in culture flasks until confluent and then transferred into Petri dishes. Retinoic acid dissolved in ethanol (Sigma; $10^{-5}\mu$M final concentration) was added to cells in the dishes. Retinoic acid was added again to the cells in the dishes when the culture medium was changed over the course of the next 5 to 7 days. The differentiated (P19N) cells were transferred into 75-cm^2 flasks or 96-well plates and retinoic acid treatment continued until the cells were used in the assay. NT2 cells were grown in culture flasks until confluent and then treated with retinoic acid dissolved in dimethyl sulfoxide ($10^{-5}\mu$M final concentration). The cells were treated with retinoic acid twice a week for a minimum of 2 weeks (4 weeks maximum). The cells were transferred into 96-well plates and retinoic acid continued until the cells were used in the assay.

HSV-1 infection

P19N and NT2N in flasks or 96-well plates were washed once with sterile phosphate-buffered saline (PBS; pH 7.4). The cells were then inoculated with either (i) sterile PBS, (ii) sterile PBS containing the appropriate amount of HSV-1 stock to reach the intended multiplicity of infection (MOI), or (iii) sterile PBS containing cell extract prepared from uninfected Vero cell cultures following the same protocol that was used for the preparation of virus stocks from HSV-1 infected Vero cell cultures (mock infection). The flasks were incubated at 37°C for 1 h with occasional gentle agitation. At the end of the incubation period, inocula used for infection or mock infection were removed and fresh culture medium was added to the cultures.

Determination of infectious HSV-1 in cultures was performed using Vero cells in standard viral plaque assays as previously described (Valyi-Nagy *et al*, 1994). In experiments that involved ebselen (2-phenyl-1,2-benzisoselanazol-3(2H)-one; Sigma-Aldrich) and HNE (4-hydroxy-2-nonenal; Calbiochem) treatment, P19N cultures were treated with 5, 10, or 25 μM of ebselen or with 5 or 50 μM of HNE for 1 h prior to and for 24 h following virus inoculation.

Immunocytochemistry

P19N cells grown on chamber slides were either inoculated with 1 PFU/cell of HSV-1 strain KOS or were mock infected with virus free Vero cell extract, incubated at 37°C for 1 h with occasional gentle agitation, washed twice in sterile PBS, and further incubated in fresh medium for 24 h. The media were then discarded and the cultures were washed twice in sterile PBS and fixed in 4 percent paraformaldehyde for 2 h. HSV-1 antigens were detected using a 1:1000 dilution of a rabbit HSV-1–specific antiserum (DAKO, Carpinteria, CA). Incubated with primary antibody at 43°C for 32 min was followed by the addition of biotinylated anti-rabbit immunoglobulin secondary antibody, avidin-horseradish peroxidase, and 3,3′-diaminobenzidine tetrahydrochloride (0.04%) in 0.05 M Tris-HCL (pH 7.4) and 0.025% H_2O_2 as a chromogen (Ventana Medical Systems, Tucson, AZ). Before staining, binding of secondary antibodies and conjugates was blocked by appropriate reagents provided by the manufacturer.

Detection of reactive oxygen radicals

These experiments were performed in 96-well assay plates (BD Falcon). P19N and NT2N cells suspended in fresh culture medium were added to each well and were then incubated overnight at 37°C in a 5% CO_2 containing atmosphere. The cells were washed once with PBS to remove unattached cells and serum. The wells were then inoculated with either HSV-1 or were mock infected or were exposed to sterile PBS. Following a 1-h incubation period at 37°C, wells were washed once with PBS, fresh culture medium without phenol red was added to each well, and plates were incubated at 37°C, 5% CO_2 for selected times. The wells were then washed once with PBS again. Hydroxyphenyl fluorescein (HPF; Molecular Probes; 5 μM final concentration) in fresh culture medium without phenol red was then added to each well to detect the presence of ROS (Setsukinai, 2003). Nonfluorescent HPF becomes fluorescent in presence of reactive oxygen species, particularly hydroxyl radicals. The plates were incubated at 37°C, 5% CO_2 for 20 to 60 min. The wells were washed once with PBS to remove any excess HPF. Fresh PBS was added to each well and the intensity of fluorescence was measured using a Tecan GENios Pro plate reader with

fluorescent excitation and emission maxima of 490 nm and 515 nm, respectively.

Measurement of lipid peroxidation with Bioxytech LPO-586 assay

These experiments were performed in 75-cm^2 tissue culture flasks (BD Falcon). P19N cells were washed once with PBS to remove unattached cells and serum. Flasks were then either exposed to (i) HSV-1 diluted in PBS, or (ii) cell extract diluted in PBS (mock infection), or (iii) PBS. The cultures were washed once with PBS after a 1-h incubation period and fresh growth medium without phenol red was added to the flasks. Aliquots of culture supernatants were taken from each flask at selected times. Butylated hydroxytoluene (ICN Biomedicals; 5 mM final concentration) was added to each aliquot to prevent further oxidation and the samples were centrifuged ($3000 \times g$, 10 min, 4°C). The aqueous portions were frozen and stored at −80°C until ready for analysis. The combined levels of MDA and 4-HAE in the samples were determined using the Bioxytech LPO-586 colorimetric assay (Oxis International) according to the manufacturer's instructions. The detected levels of MDA and 4-HAE were normalized to the amounts of total protein measured in the samples. Total protein in each sample was determined using the Coomassie Plus Protein Assay Kit (Pierce) according to the manufacturer's instructions. All measurements were made using a Biomate 3 spectrophotometer.

Statistical analysis

Data were analyzed using a T-test for independent samples (Vassar Stats). A value of $P < .05$ was considered significant.

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