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Short Communication

Caspase-3–dependent reactivation of latent herpes simplex virus type 1 in sensory neuronal cultures

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Life-long latent herpes simplex virus type 1 (HSV-1) is harbored in sensory neurons where sporadic reactivation occurs. Reactivation stimuli may involve activation of apoptotic signaling in the neuron. Previous experiments have demonstrated that reactivation of latent HSV-1 in dorsal root ganglion (DRG) neuronal cultures occurred following nerve growth factor (NGF) deprivation. NGF deprivation stimulates apoptotic signaling by activating the proapoptotic proteolytic enzyme, caspase-3. When DRG neuronal cultures harboring latent HSV-1 were treated with a caspase-3–specific inhibitor, NGF deprivation–induced reactivation was significantly reduced. Interestingly, the caspase-3 inhibitor had no effect on productive HSV-1 infection. Furthermore, activation of caspase-3 with either C2-ceramide or a recombinant adenovirus expressing caspase-3 caused significant HSV-1 reactivation. *Journal of NeuroVirology* (2003) **9,** 390–398.

Keywords: apoptosis; caspase-3; dorsal root ganglion; HSV-1; latency

Introduction

Herpes simplex virus type 1 (HSV-1) is classified as an Alphaherpesvirinae and it is a linear, double-stranded DNA virus containing an envelope and an icosahedral-shaped nucleocapsid. HSV-1 primary infections manifest as fever blisters on mucous membranes or skin (reviewed by Bader *et al*, 1978). A more severe effect of the virus includes infection of the corneal epithelium, the leading infectious cause of blindness (Cook, 1992). Furthermore, HSV-1 infection in the brain results in encephalitis, a serious condition leading to high mortality of the infected individual.

Following a primary infection, HSV-1 gains access to the peripheral nervous system where it establishes a latent infection. Sensory neurons in the dorsal root ganglion (DRG) and trigeminal ganglion are the primary site of HSV-1 latency in humans (Stevens, 1989). The typical recurrence of herpes infections is most often attributed to reactivation of the latent, life-long persistent infection (Wagner and Bloom, 1997).

HSV-1 gains access to nerve termini during the productive or primary infection of mucocutaneous membranes. The virus moves from the neuronal terminal to the cell body via retrograde transport to establish a latent infection (Baringer and Swoveland, 1973). Following various stimuli, including stress, fever, ultraviolet (UV) irradiation, and trauma to the skin, HSV-1 can sporadically reactivate. Upon reactivation, anterograde transport of newly formed infectious HSV-1 viral particles travel back to the dermis to reinfect and repeat the cycle of infection. To date, there is no known pharmacological agent capable of completely eliminating reactivation or preventing the establishment of latency. Determining the cellular factors that cause reactivation of latent HSV-1 within the sensory neuron cell bodies of the dorsal root and trigeminal ganglion cells is critical for the design and development of effective pharmacological agents for the treatment of sporadic reactivation. The experiments presented here examine the potential role of apoptotic signaling pathways induced following a reactivation stimulus and, more specifically, the role of caspase-3 as a host cellular factor involved in HSV-1 reactivation following a latent infection.

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We have developed an *in vitro* model to study HSV-1 latency and reactivation in sensory neurons in culture (Wilcox and Johnson, 1988). In this model, primary DRG cultures are latently infected with HSV-1 and reactivation is induced by various stress stimuli, including nerve growth factor (NGF) deprivation (Wilcox and Johnson, 1987). During latency, there is no detectable infectious viral particles present and only following a reactivation stimulus can infectious virus be assayed (Wilcox and Johnson, 1988). Similar to latent infections *in vivo*, HSV-1 is detected in the neuronal cultures as an episome in the nucleus and stain positive for the latency-associated transcript (LAT) (Smith *et al*, 1994b). This *in vitro* neuronal model provides a means to study HSV-1 latency and reactivation in a controlled and defined environment (Doerig *et al*, 1991; Wilcox and Johnson, 1988; Wilcox *et al*, 1990).

Reactivation stimuli for latent HSV-1 in the *in vitro* latency model was initially characterized utilizing NGF withdrawal. In the developing nervous system, NGF is an important neurotrophic factor necessary for the development, growth, survival, maintenance, and repair of the sensory neuron (Hamburger and Levi-Montalcini, 1949). These effects are mediated through two receptors that bind NGF, TrkA (tyrosine kinase A) and p75NGF. The TrkA receptor is essential for cell survival and the p75NGF receptor appears to signal apoptosis (Barrett and Bartlett, 1994; Kaplan *et al*, 1991; Itoh *et al*, 1991; Martin-Zanca *et al*, 1986). *In vitro*, NGF deprivation often induces an apoptotic signal transduction pathway in immature sensory neurons (reviewed by Kaplan and Miller, 2000). The mechanism of apoptosis following NGF deprivation is complex and may involve the lack of signaling of the TrkA receptor and/or the initiation of p75NGF receptor signaling.

The p75NGF receptor is in the same receptor family as the tumor necrosis factor-alpha (TNF-μ)R1 and the Fas/Apo-1 receptors, suggesting that these receptors share similar apoptotic signaling (Smith *et al*, 1994a). Both the TNF-μR1 and Fas/Apo-1 receptors contain a cytoplasmic death domain region capable of binding adaptor molecules, such as caspase-8, ultimately resulting in activation of the downstream target, caspase-3 (Boldin *et al*, 1996). Caspases or cysteinyl aspartate–specific proteases are characterized as important activators of the apoptotic signal transduction pathway possibly via the p75NGF receptor following NGF deprivation in neurons.

Activation of the caspases initiates an enzymatic cascade often resulting in apoptosis (reviewed by Kumar and Colussi, 1999). Apoptosis is an orderly process where cell death occurs as sequential enzymatic reactions ultimately ending in the fragmentation of DNA and condensation of the cell. This process is especially important in the developing nervous system to attain proper neuronal connections. The importance of apoptosis in the nervous system became evident following the severely altered neuronal development in the caspase-3–knockout mouse. These animals developed an enlarged nervous system and were severely compromised by the lack of normal developmental neuronal apoptosis (Kuida *et al*, 1996).

Caspase-3 is activated from the proenzyme form by caspase-8. Once proteolytic cleavage of caspase-3 occurs, the activated enzyme translocates to the nucleus to cleave its substrate target proteins including the DNA repair enzyme, poly(ADP ribose) polymerase (PARP) and DNA-protein kinase (DNA-PK). The ultimate downstream events following caspase-3 activation include DNA fragmentation and chromatin condensation, which are hallmarks of the end stages of apoptosis.

Previous studies indicated that caspase-3 may affect productive HSV-1 infection in cell lines (Galvan *et al*, 2000; Galvan and Roizman, 1998; Jerome *et al*, 1999). Our studies focus on the relevance of caspase-3 activation in the initiation of HSV-1 reactivation following a latent infection in sensory neurons. In order to investigate the role of caspase-3 during NGF deprivation–induced reactivation of latent HSV-1, we used several approaches, including a specific inhibitor to caspase-3, caspase-3 activation, and overexpression of caspase-3 with an adenoviral vector.

Caspase-3 inhibitor attenuates HSV-1 reactivation following NGF deprivation but does not prevent reactivation induced by forskolin or phorbol 12-myristate 13-acetate (PMA) treatment

HSV-1 alters apoptotic pathways during a productive infection *in vitro* in many different cell lines, but its effects on apoptosis during a latent infection in neurons is unclear (Aubert and Blaho, 1999; Galvan *et al*, 2000; Jerome *et al*, 1999; Munger *et al*, 2001; Zhou and Roizman, 2000). Previously, our laboratory demonstrated that NGF deprivation initiated HSV-1 reactivation from latently infected DRG neurons in culture (Wilcox and Johnson, 1988). In order to determine the signaling pathway involved in this reactivation stimulus, we studied caspase-3, a possible downstream pathway. In immature neurons not infected with HSV-1, NGF deprivation initiates an apoptotic-signaling pathway often dependent on caspase-3 (reviewed by Kaplan and Miller, 2000). However in mature DRG neurons, NGF deprivation does not induce apoptosis but may still induce caspase-3 activation (Volgelbaum *et al*, 1998). Experiments in this study uncover the relevance of caspase-3 following reactivation induced by NGF deprivation in HSV-1 latently infected mature DRG neurons.

Previous characterization of the *in vitro* latency model determined that NGF withdrawal created a stress response in the neurons sufficient to induce HSV-1 reactivation (Wilcox and Johnson, 1988). Sensory neuronal cultures were prepared from DRG of

embryonic day 15 Sprague-Dawley rats as previously described (Smith and Wilcox, 1996; Wilcox *et al*, 1990). Neurons were plated onto 24-well plates at a cell density of (1–5) $\bigcirc 10^3$ cells per well. Dulbecco's modified Eagle's/F12 medium supplemented with 10% newborn bovine serum and 100 ng/ml 2.5 S NGF (Harlan Bioproducts Indianapolis, IN) was used to maintain neuronal cultures (neuronal maintenance medium). Cultures were treated with fluorodeoxyuridine (20 μM) for 7 to 10 days after plating to reduce the non-neuronal cell population.

Latent HSV-1 infection in DRG cultures was established as previously described (Smith and Wilcox, 1996; Wilcox and Johnson, 1988; Wilcox *et al*, 1990). Briefly, after neurons were allowed to mature in tissue culture for 21 days, they were pretreated with 50 μM acycloguanosine (Sigma, St. Louis, MO) 24 h prior to inoculation with HSV-1 (17+ strain) and for 7 days following inoculation. Neuronal cultures were infected with approximately 10 plaque-forming units (pfu) of HSV-1 (17^+) per neuron for 1 h followed by the removal of the virus, washing the cells, and replacement of the neuronal maintenance medium. Once a latent infection was established, the acycloguanosine was removed. The neurons were latent for 14 days where no infectious virus was detected in either the cell extract or the medium during the latent infection (Wilcox and Johnson, 1988).

In order to determine whether apoptotic pathways were activated in this model, we pretreated the DRG neurons with a tetrapeptide analog inhibitor of caspase-3, DEVD-CHO (Calbiochem, La Jolla, CA), prior to reactivating with NGF withdrawal. This inhibitor is a specific tetrapeptide analog of the cleavage site of caspase-3, thus the enzyme cannot be cleaved to its active form. Reactivation was induced by washing the cells three times with medium containing no NGF and subsequent treatment with anti-NGF polyclonal rabbit antibody for 5 days. The caspase-3 inhibitor was replenished in the medium 48 h following the initial reactivation stimulus. Control cultures were treated the same as reactivated cultures except NGF was not removed. Additionally, control cultures were treated with DMSO as a vehicle control at similar concentrations as 10 nM DEVD-CHO–treated cultures. Occasionally sporadic reactivation can occur in control cultures attributed to the rigorous wash cycles necessary to remove any NGF from the cultures. We analyzed the culture medium and cell extract for infectious virus following reactivation stimulus and performed plaque assays in Vero cells (American Type Culture Collection [ATCC], Rockville, MD) to determine viral titers for each treatment group. Briefly, Vero cells were plated on 24-well plates and incubated with a 200-μl aliquot of the medium and cell extract from reactivated DRG cultures. Because HSV-1 is a cell-associated virus, cellular extract in conjunction with the cell medium was used for the plaque assays and referred to as DRG extract. Plaque formation in the Vero cells occurred within 48 h post infection with the DRG extract. The plaques were counted to determine viral titers for each well. Each experiment consisted of an *n* of 6 for each treatment group and was repeated three to five times. The data were analyzed using a one-way analysis of variance (ANOVA) with SAS statistical software.

NGF deprivation–induced HSV-1 reactivation resulted in a mean of 76,500 pfu/ml, whereas the cultures treated with the caspase-3 inhibitor had an average of 39,000 pfu/ml for 1 nM and 46,700 pfu/ml for 10 nM DEVD-CHO. Neurons treated with the caspase-3 inhibitor exhibited on average a two-fold reduction in viral titers relative to the untreated NGF-deprived neurons, which was statistically significant (1 nM, $P = .02$; 10 nM, $P = .015$) (Figure 1). This indicated that caspase-3 activation was important for efficient HSV-1 reactivation induced by NGF deprivation. However, the caspase-3 inhibitor was unable to completely abolish HSV-1 reactivation induced by NGF deprivation, due to several potential factors. Viral reactivation via NGF deprivation is a slow response, requiring 4 to 5 days to recover infectious virus from the DRG cultures. Because the inhibitor is a tetrapeptide analog, some of the caspase-3 inhibitor may have been inactivated during the course of reactivation. In addition, other pathways, besides caspase-3, may be activated in response to NGF deprivation that are important modulators of reactivation. Caspase-3 may play a partial role in reactivation in the mature neuron due to the complexity of NGF signaling, mediated via two receptors, with several signaling pathways initiated through each of these receptors. Furthermore, following developmental maturation, the neuron loses its dependence on NGF for survival, suggesting that the apoptotic pathway that is functional during neuronal development is only partially functional. Because the DRG neurons were allowed to mature for 21 days, they have acquired this independence from NGF for survival, which may explain the partial protection provided by the caspase-3 inhibitor.

In order to determine whether caspase-3 inhibitor effect on HSV-1 reactivation was specific for NGF deprivation, we tested its effects following HSV-1 reactivation with PMA and forskolin. These two pharmacological agents induce HSV-1 reactivation but not apoptosis. Previous data had indicated the activation of protein kinase C (PKC) with PMA or activation of protein kinase A (PKA) using forskolin-induced reactivation of HSV-1 in latently infected neurons (Smith *et al*, 1992). The DRG cultures were reactivated with 100 μM forskolin (Sigma, St. Louis, MO) and with PMA (Sigma) at $10^{\bigcirc 9}$ M for 3 days. The caspase-3 inhibitor did not prevent HSV-1 reactivation when added prior to forskolin treatment. Similar results were observed following PMA-induced HSV-1 reactivation. This indicates that caspase-3 inhibition did not prevent reactivation induced by forskolin or PMA (Figure 2). Therefore, it appears that caspase-3 inhibition only affect reactivation initiated by NGF

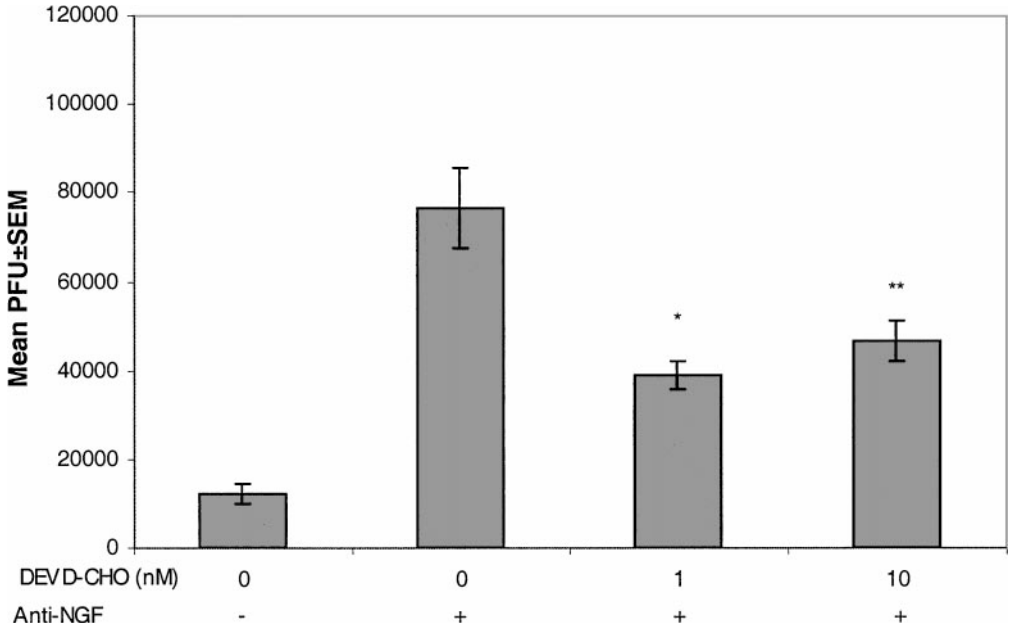


Figure 1 NGF withdrawal-induced HSV-1 reactivation in latently infected DRG neurons was attenuated by treatment with caspase-3 inhibitor. DRG cultures were latently infected with HSV-1 (17+) at an MOI = 10 for 14 days, followed by reactivation with NGF withdrawal. Each treatment group consist of $n = 18$. SAS statistical software analysis using a one-way ANOVA with a Tukey-Kramer adjustment determined the statistical significance of the caspase-3 inhibitor at concentration of 1 nM, $^{*}P = .02$, and 10 nM, $^{**}P = .015$, compared to control NGF withdrawal.

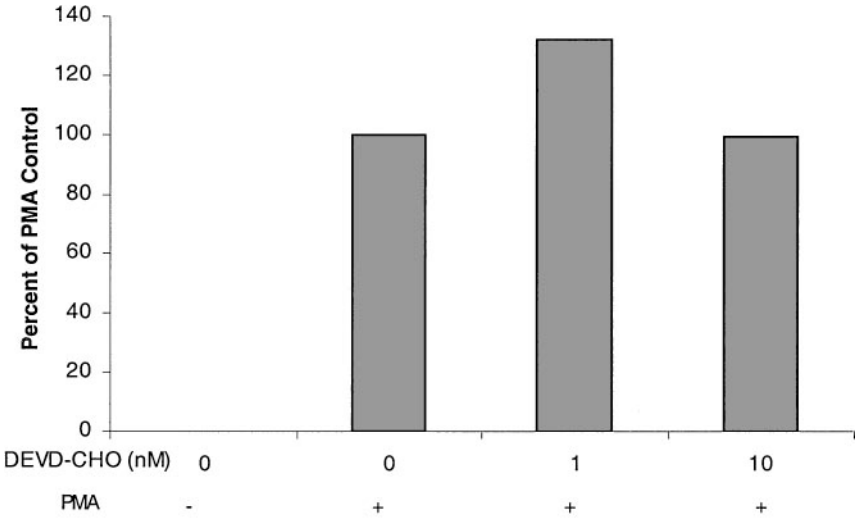
withdrawal and does not affect reactivation initiated by PMA and forskolin.

Caspase-3 inhibitor had no effect on productive HSV-1 infection in neuronal cultures

Previous studies have shown that the caspase-3 inhibitor affects productive infection in cell lines (Galvan and Roizman, 1998). We tested the effects of the caspase-3 inhibitor in a productive infection in primary DRG cultures. In order to establish a productive infection in the DRG neurons, the neurons were infected at a higher multiplicity of infection (MOI) of 50 pfu per neuron for 1 h. Following incubation for 1 h, the virus was removed and the cells were washed to remove any excess virus that was not absorbed after the incubation period. To determine the effects of the caspase-3 inhibitor on productive infection, the DRG neurons were pretreated with 1, 10, and 100 nM caspase-3 inhibitor, DEVD-CHO (Calbiochem) for 2 h prior to infection and re-treated following removal of virus and wash. The viral titers were assessed 24 h following the initial infection using a standard plaque assay in Vero cells as previously described. Our results determined that inhibition of caspase-3 had no effect on a productive infection in the DRG cultures. Control cultures included no treatment and vehicle control with DMSO concentration similar to 100 nM DEVD-CHO treatment group. HSV-1 viral titers were used to assess the effects of the inhibitor on the production of newly synthesized infectious virus. The data indicated no statistical significance between the caspase-3 inhibitor and the untreated controls (Figure 3).

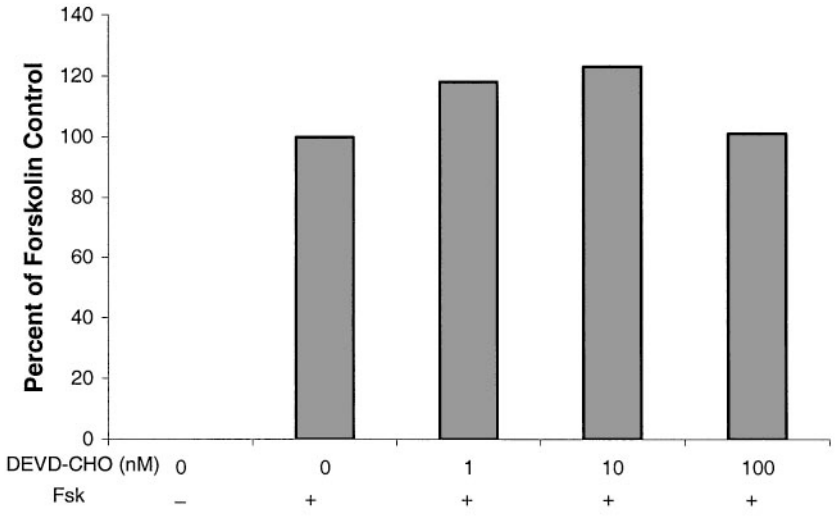
Previously published data indicate that HSV-1 is both dependent and independent of caspase-3 activation during a productive infection in actively dividing cell lines (Galvan *et al*, 2000). Munger *et al* (2001) presented results that implicate viral protein US3 as a caspase-3 inhibitor. The data presented here demonstrate that in DRG neurons the caspase-3 inhibitor had no effect on productive HSV-1 infection. Therefore, the effects of the caspase-3 inhibitor were specific for HSV-1–induced reactivation from a latent infection. However, the targeted protein for caspase-3 proteolysis is still unknown and may involve either an indirect effect through cellular targets or a direct viral target.

It is possible that caspase-3 is important during a productive infection in non-neuronal cells to maintain an appropriate level of ICP4 and ICP27 that promotes viral gene transcription. Because ICP4 and ICP27 both have caspase-3–targeted cysteine-aspartate sequences, they may compete with caspase-3 host cellular targets and therefore prolong cell survival and ensure the progression from alpha to beta gene transcription. Mutant studies verify that in the absence of ICP4 and ICP27, the cell is not protected from apoptosis (Aubert *et al*, 1999; Aubert and Blaho, 1999; Galvan and Roizman, 1998). It is possible that HSV-1 requires the breakdown of ICP4 and ICP27 and relies on host apoptotic enzyme activity



Treatment	Mean pfu/ml ± SEM
0	0
PMA	925 ± 51
PMA + 1nM DEVDCHO	1220 ± 61
PMA + 10nM DEVDCHO	920 ± 63

(**A**)



Treatment	Mean pfu/ml ± SEM
0	0
Fsk	426,000 ±37,142
Fsk + 1nM	505,000 ±89,183
Fsk + 10nM	522,500 ±90,000
Fsk + 100nM	430,000 ±51,020

(**B**)

Figure 2 The caspase-3 inhibitor did not affect HSV-1 reactivation with PMA or forskolin. (**A**) DRG latently infected neurons were pretreated with caspase-3 inhibitor (DEVD-CHO) prior to reactivation with 100 μM forskolin. Controls included no-treatment latent neurons and reactivated neurons with forskolin alone. Each group represents $n = 6$. The data are represented as a percentage of positive-control cultures. Actual mean pfu/ml for each treatment is represented in the tables below each graph. A one-way ANOVA determined no statistically significant difference between caspase-3 inhibitor–treated groups and the forskolin-treated positive control. (**B**) The caspase-3 inhibitor had no effect following 1 ◯$10^{\bigcirc 9}$ M 13-phorbol-12-myrestate acetate (PMA)-induced HSV-1 reactivation of latent DRG cultures. The data are represented as a percentage of PMA-treated cultures. Each group represents $n = 6$ and SAS analysis using a one-way ANOVA determined no significant differences between caspase-3 inhibitor groups to the PMA-treated positive control. Actual mean pfu/ml are present in the table below the graph.

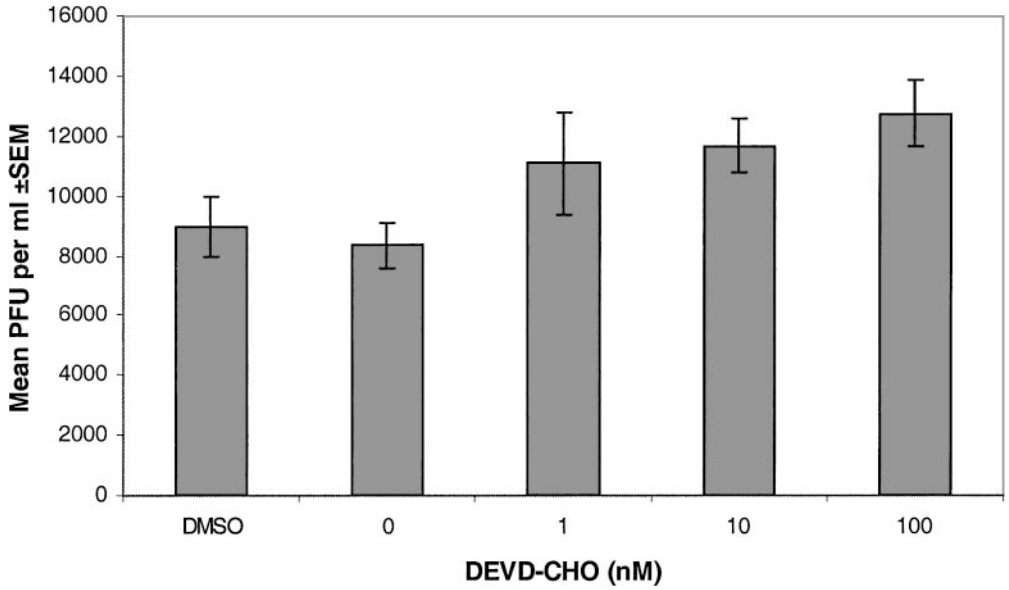


Figure 3 Caspase-3 inhibitor did not affect viral replication during a productive infection in DRG cultures. The neurons were pretreated with the caspase-3 inhibitor (DEVD-CHO) prior to HSV-1 infection at an MOI = 50. Viral titers were assessed at 24 h post infection. One-way ANOVA showed no significant differences between treatment groups. Each group consisted of $n = 6$.

for replication efficiency during a productive infection in actively dividing cells.

Activation of caspase-3 by C2-ceramide–induced reactivation of latent HSV-1

In addition to demonstrating that inhibitors of caspase-3 diminished reactivation in cultured DRG neurons, we wished to determine whether activators of caspase-3 enhanced reactivation of HSV-1 latently infected DRG cultures. In the absence of NGF, p75NGF causes the synthesis of C2-ceramide, which activates caspase-3, resulting in HSV-1 reactivation (Gill and Windebank, 2000; Brann *et al*, 2002).

C2-ceramide is generated following the hydrolysis of sphingomyelin by the activation of the enzyme sphingomylinase. During apoptosis, levels of C2-ceramide correlate with caspase-3 cleavage and activation. By using cell-permeable C2-ceramide, we directly tested the effects of C2-ceramide on caspase-3 activation and confirmed that this caspase-3–dependent pathway induces reactivation of latent HSV-1 (Figure 4). C2-ceramide (Sigma) was tested at concentrations of 25 and 50 μM and incubated for 3 days. C2-ceramide caused a significant dose-dependent HSV-1 reactivation with viral titer levels of 1500 pfu/ml at 10 μM, 9100 pfu/ml for 25 μM, and 18,150 pfu/ml for 50 μM (Figure 4). There was an overall 54-fold increase in viral titers at 50 μM of C2-ceramide compared to control cultures, which was statistically significant ($P = .0054$). In conclusion, these data suggest that caspase-3 activation can directly cause HSV-1 reactivation from a latent infection.

Caspase-3 activation in mature neurons after NGF deprivation may involve signaling through the low affinity p75NGF receptor. The p75NGF receptor signals through two pathways that activate caspase-3 indirectly. First, p75NGF activates the caspase enzymes through a putative caspase-8 adaptor molecule attached to the internal domain of the receptor. It has been postulated that the p75NGF receptor behaves as a NGF sensor, determining the available amount of NGF present for the cell (Rabizadeh *et al*, 1993). Therefore, during NGF deprivation, the lack of binding of NGF to the p75NGF receptor may initiate proteolytic activation of caspase-3 either via C2-Ceramide or caspase-8.

The recombinant adenovirus expressing caspase-3–induced reactivation in HSV-1 latently infected neurons

To further test the hypothesis that direct activation of caspase-3 induces HSV-1 reactivation from latently infected DRG neurons, we overexpressed caspase-3 using an adenovirus vector. Because DRG neurons cannot be efficiently transfected, we used an adenovirus to express caspase-3. Adenovirus vectors have been previously used to efficiently introduce and express transgenes in DRG neurons *in vitro* without cytotoxic effects (Colgin *et al*, 2001; Smith *et al*, 2000). In order to construct the adenoviral vector, we used pET-23b plasmid backbone containing human caspase-3 (CPP32) complete coding sequence

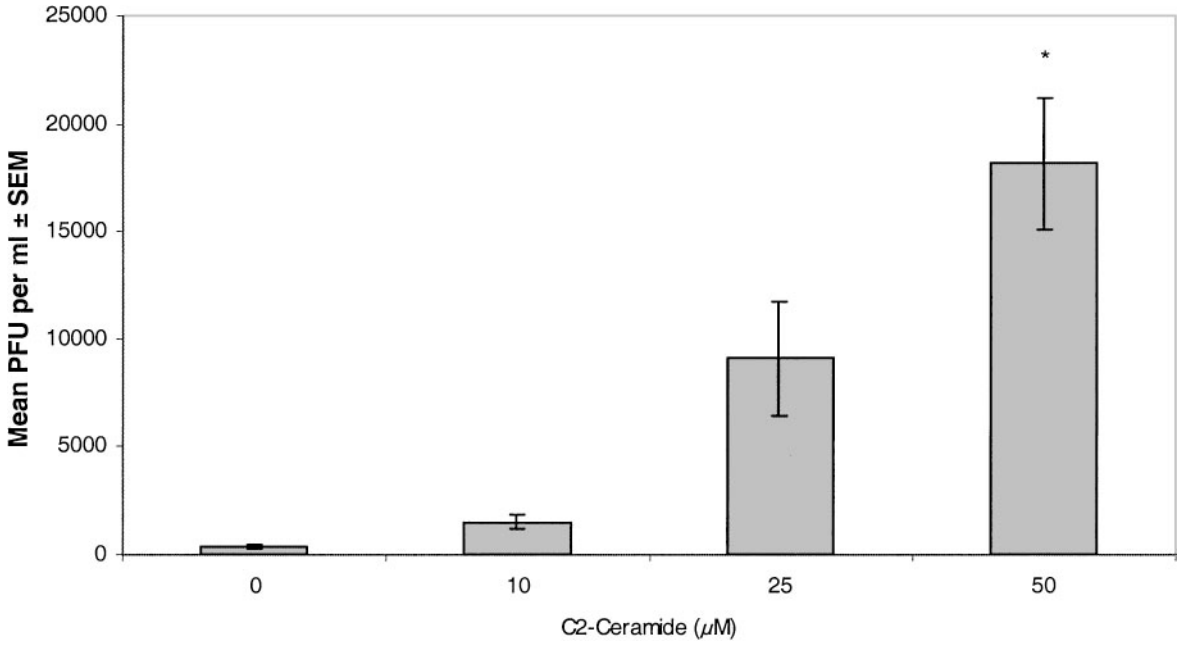


Figure 4 C2-ceramide–induced HSV-1 reactivation in latently infected DRG cultures. Latently infected DRG neurons were reactivated with C2-ceramide for 4 days and viral titers were assayed. The data are representative of five separate experiments with $n = 18$ for each treatment group. Analysis using an ANOVA determined that viral titers obtained from reactivation at 50 μM C2-ceramide were statistically significant from control cultures. $\mathcal{P} = .0054$.

purchased from ATCC (catalogue no. 99625, PubMed 96355448). CPP32 was excised from the pET-23b using NdeI and XhoI and subcloned into the left end of the adenovirus plasmid (E1A promoter) to create pE1A-CPP32. Both the pE1A-CPP32 and pJM17 were cotransfected into 293 cells to generate the recombinant adenoviral vector, Ad-E1A-CPP32. The adenovirus used in these studies is replication deficient. Ad-E1A-CPP32 titers were obtained using a standard plaque assay in 293 cells. MOI of approximately 50 and 100 were used to infect the DRG cultures. The latently infected DRG cultures were infected with Ad-E1A-CPP32 for 5 days in order to obtain maximal expression of caspase-3. Following infection with Ad-E1A-CPP32, we observed a robust HSV-1 reactivation of the latently infected DRG cultures at both MOI of 50 and 100 (Figure 5). The adenoviral control consisting of an adenovirus expressing green fluorescent protein (GFP) demonstrated no viral reactivation from latently infected DRG cultures. HSV-1 viral titers were determined using a standard plaque assay in Vero cells as previously described. Viral titers from reactivated DRG cultures at MOI = 50 was 10,630 pfu/ml, whereas at an MOI = 100, the viral titers were 98,040 pfu/ml, a ninefold increase; therefore, an increase in MOI correlated with an increase in HSV-1 viral titers. In conclusion, the overexpression of caspase-3 in the latently infected DRG cultures induces HSV-1 reactivation.

The induction of HSV-1 reactivation through the activation of caspase-3 may be relevant towards understanding the role of the LAT (Perng *et al*, 2000). Recent evidence demonstrated that LAT has anti-apoptotic activity (Inman *et al*, 2001; Ahmed *et al*, 2002). Studies by Inman *et al* (2001) mapped the regions within the LAT transcript that inhibits apoptosis in tissue culture and promotes neuronal cell survival in an *in vivo* ocular rabbit model of HSV-1 latency. Furthermore, these studies suggest a direct interaction of LAT with members of the apoptotic pathway, including the pro-apoptotic protein Bax (Inman *et al*, 2001). These studies demonstrate that HSV-1 utilizes the anti-apoptotic activity of LAT to promote cell survival following the induction of apoptosis in non-neuronal cells as well as the survival of neurons *in vivo*. Our studies imply that a stress stimulus sufficient to overcome the anti-apoptotic activity of LAT and induce apoptotic pathways in neurons involving caspase-3 activation ultimately results in HSV-1 reactivation *in vitro*.

In conclusion, our experiments show that a specific caspase-3 inhibitor attenuates NGF deprivation–induced HSV-1 reactivation. This appears to be specific for NGF-induced reactivation, because the caspase-3 inhibitor did not affect HSV-1 reactivation following treatment with forskolin or PMA, two pharmacological agents known to cause reactivation in this *in vitro* model, but do not cause apoptosis (Smith *et al*, 1992). Furthermore, the caspase-3 inhibitor does not affect viral replication during a productive infection in neurons. Caspase-3 activation appears to have a direct effect on latent HSV-1 because C2-ceramide, an activator of caspase-3 *in vitro*, caused HSV-1 reactivation. Moreover, the recombinant

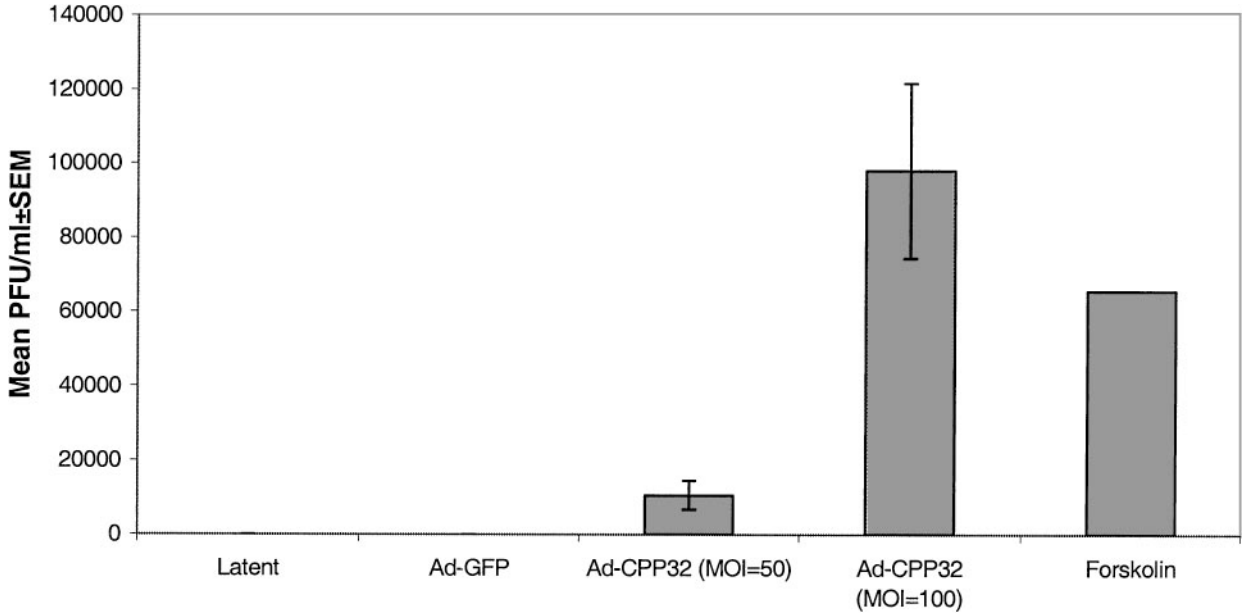


Figure 5 Recombinant adenovirus expressing caspase-3 (Ad-E1A-CPP32) induced an MOI-dependent HSV-1 reactivation in latently infected DRG cultures. HSV-1 latently infected DRG neurons were treated with Ad-E1A-CPP32 and viral titers for reactivated HSV-1 were assayed. Controls include latent cultures not treated with Ad-E1A-CPP32, Ad-GFP, and a positive control of 100 μM forskolin.

adenovirus expressing caspase-3 confirmed this direct role of caspase-3 in the induction of reactivation of HSV-1 latently infected cultures. Taken together, these experiments show a direct effect of caspase-3, a component of the apoptosis signal pathway, as an important mediator for HSV-1 reactivation.

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