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Resveratrol suppresses nuclear factor-κB in herpes simplex virus infected cells

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Abstract

Resveratrol inhibits herpes simplex virus (HSV) replication by an unknown mechanism. Previously it was suggested that this inhibition may be mediated through a cellular factor essential for HSV replication [Docherty, J.J., Fu, M.M., Stiffler, B.S., Limperos, R.J., Pokabla, C.M., DeLucia, A.L., 1999. Resveratrol inhibition of herpes simplex virus replication. Antivir. Res. 43, 145–155]. After examining numerous cellular factors, we report that resveratrol suppresses NF-kappaB (NF-κB) activation in HSV infected cells. Reports have indicated that HSV activates NF-κB during productive infection and this may be an essential aspect of its replication scheme [Patel, A., Hanson, J., McLean, T.I., Olgiate, J., Hilton, M., Miller, W.E., Bachenheimer, S.L., 1998. Herpes simplex type 1 induction of persistent NF-kappa B nuclear translocation increases the efficiency of virus replication. Virology 247, 212–222; Gregory, D., Hargett, D., Holmes, D., Money, E., Bachenheimer, S.L., 2004. Efficient replication by herpes simplex virus type 1 involves activation of the IkappaB kinase-IkappaB-RelA/p65 pathway. J. Virol. 78, 13582–13590]. Electromobility shift assays determined that resveratrol, in a dose dependent and reversible manner, suppressed activation of NF-κB in Vero cells infected with HSV-1, HSV-2 and acyclovir resistant HSV-1. Furthermore, resveratrol did not protect IκBα, a cytoplasmic NF-κB inhibitor, from degradation in HSV-1 infected cells. Immunohistochemical studies demonstrated that RelA/p65, a component of the dimeric NF-κB complex, translocated to the nucleus of HSV-1 infected cells in the presence of resveratrol. Finally, direct effects on viral transcription and DNA synthesis were evaluated. Real-time RT-PCR analysis showed that resveratrol treatment of infected cells resulted in reductions of mRNA for ICP0, ICP4, ICP8 and HSV-1 DNA polymerase by 2.1-, 3.3-, 3.8- and 3.1-fold, respectively. Plus, mRNA for glycoprotein C, an HSV late gene, was completely absent in the presence of resveratrol. Lastly, quantitative PCR showed that resveratrol significantly blocked HSV DNA synthesis. Cumulatively, these data indicate that resveratrol (i) suppresses HSV induced activation of NF-κB within the nucleus and (ii) impairs expression of essential immediate-early, early and late HSV genes and synthesis of viral DNA.

Keywords: Resveratrol; Herpes; NF-κB; Suppression

1. Introduction

Herpes simplex virus (HSV) typically causes benign lesions, such as cold sores, but is capable of causing serious afflictions, such as genital herpes, keratitis and neonatal encephalitis (Corey et al., 1983; Cobo, 1988; Whitley, 1993). Due to the large number of individuals infected with this virus and because the infection is for life, improving on existing therapies is an ongoing endeavor. One such drug under investigation is resveratrol (3,4′,5-trihydroxystilbene), a polyphenol produced by some spermatophytes, such as grapes and peanuts in response to stress and fungal infection (Langcake and Pryce, 1977; Sanders et al., 2000). This chemical has been shown to inhibit HSV-1 and HSV-2 in vitro in a dose and time dependent manner (Docherty et al., 1999). Additionally, in vivo, topically applied resveratrol significantly reduced lesion development and mortality rates of mice dermally or vaginally infected with HSV (Docherty et al., 2004, 2005). However, the mechanism by which resveratrol exerts antiviral effects on HSV replication is unknown.

Therefore, studies were designed to determine the mechanism(s) by which resveratrol inhibits HSV replication. Previous studies suggested that resveratrol was affecting the host cell in

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a non-lethal manner, indicated by induction of cell cycle arrest at the G2/M interphase (Docherty et al., 1999). Numerous cellular factors essential to HSV replication that control the G2/M progression were examined: cyclin dependent kinase 1 (cdk1), cyclins A and B, p53, MDM2 and p21 (data not reported). Data from these experiments yielded insufficient proof that these factors were affected by resveratrol treatment in HSV infected cells. Thus, attention was focused onto another cell factor that has a role in mitigating cell cycle activities, nuclear factor kappaB (NF-κB).

NF-κB is a host nuclear transcription factor, activated by multiple stimuli, including inflammatory cytokines, growth factors and bacterial or viral infections (Santoro et al., 2003). Interestingly, NF-κB has been shown to be a cellular factor activated in response to HSV infection (Patel et al., 1998; Amici et al., 2001; Goodkin et al., 2003; Taddeo et al., 2003; Gregory et al., 2004) and at least two immediate-early herpes proteins, ICP4 and ICP27, have a demonstrated role in the activation of NF-κB (Margolis et al., 1992; Patel et al., 1998). Recently, NF-κB was reported to interact directly with the HSV genome by binding to the promoter of ICP0, an immediate-early gene (Amici et al., 2006). Interestingly, others have reported that resveratrol inhibits activation of NF-κB in model systems (Manna et al., 2000; Takada et al., 2004). In our studies reported on here, we were able to demonstrate that NF-κB was adversely affected by resveratrol in HSV infected cells, which could account for the inhibitory effects on HSV replication.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney cells (Vero) were acquired from the American Type Culture Collection (Rockville, MD) and grown in complete media (Media 199 supplemented with 5% fetal bovine serum, 0.225% $NaHCO_3$, 25 mM HEPES pH 7.2 and 50 μg/ml gentamycin sulfate). HSV-1 and HSV-2 (Zimmerman et al., 1985) or acyclovir resistant HSV-1 (ACV-R HSV-1; Parris and Harrington, 1982) were used in studies as follows: Vero cells were infected with HSV at a multiplicity of infection (moi) of 1, 5 and 10 and allowed to adsorb for 1 h at 37 °C. The cell lawn was washed twice with complete media without drug to remove unadsorbed virus and then drug containing media or control media was added. At indicated times post infection, samples were harvested according to experimental protocols.

2.2. Chemicals and reagents

Resveratrol (3,4′,5-trihydroxy-trans-stilbene), (Royalmount Pharma Inc. Montréal, Que.), was dissolved in DMSO to form stock solutions and protected from light at all times. Stock solutions were diluted in complete media, resulting in the final desired concentration of drug and 0.2% DMSO. Prior analysis of Vero cells by trypan blue viability and MTT cytotoxicity assays generated a sigmoidal viability curve that indicated the CD_{50} of resveratrol 48 h post exposure was 355 μM, while 219 μM resveratrol did not induce cellular toxicity. For control media, DMSO was added to complete media at a final concentration of 0.2%, which had no effect on HSV replication or cell viability. For viral DNA studies, acyclovir (Sigma–Aldrich, St. Louis, MO), was diluted in sterile water and added to infected cells at an inhibitory concentration of 10 μM after the 1 h adsorption period.

Antibodies against IκBα (Cell Signaling Technologies, Beverly, MA), β-actin (Sigma–Aldrich), RelA/p65, anti-rabbit immunoglobulin-conjugated with peroxidase, anti-mouse immunoglobulin-conjugated with peroxidase (Santa Cruz, Santa Cruz, CA) and ICP4 (Rumbaugh-Goodwin Institute, Plantation, FL) were employed in Western blots. A protease inhibitor cocktail (Sigma–Aldrich) was used in all cellular extracts.

2.3. Electromobility shift assays (EMSA)

Vero cells in 25 cm^2 flasks (1.5×10^6 cells) were infected and treated as described. At indicated times, cells were washed and scraped into cold PBS and centrifuged at 600 × *g* for 5 min. Nuclear extracts were obtained by a modified Dignam procedure (Dignam et al., 1983). 150 μl DA buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM DTT, 0.2% Igepal CA630, 1 mM NaF, 1 mM Na_3VO_4 and 1:100 protease inhibitor cocktail) was added to cell pellets, which were vortexed for 15 s and placed on ice for 10 min. 10 μl of 10% Igepal CA630 (Sigma–Aldrich) was added and the samples were incubated for 1 additional minute. Samples were centrifuged at 16,000 × *g* for 5 min at 4 °C and the cytoplasmic supernatant was removed. Nuclear pellets were lysed for 40 min and vortexed 15 s every 10 min in 75 μl DB buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 and 1:100 protease inhibitor cocktail). Samples were centrifuged at 16,000 × *g* for 10 min at 4 °C and aliquots of the nuclear supernatant frozen at −80 °C until assayed. Next, the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) was used according to manufacturer's protocol. Briefly, nuclear extracts were incubated for 20 min with a biotin end labeled duplex DNA κB response element, 5′-biot-AGTTGAGGGGACTTTCCCAGGC-3′ (Cameron et al., 2002), at 25 °C and electrophoresed in non-denaturing 6% PAGE. DNA was capillary transferred to a nylon membrane, UV-cross-linked and detected by chemiluminescence. Band densitometry of radiographic films was calculated with Total Lab v2003.02 software.

2.4. Biotin–streptavidin pull-down assay

The ability of NF-κB to bind to the duplex κB response element was examined using a pull-down assay (Ragione et al., 2003). Briefly, 180 μg of nuclear extract from HSV-1 infected Vero cells 18 h post infection was incubated with a non-specific biotinylated probe provided with the LightShift EMSA Kit (Pierce) or with the biotinylated κB response element with or without an identical non-biotinylated duplex in 200:1 molar excess using the conditions described in the EMSA assay above. The DNA–protein complexes were precipitated and purified

using pre-adsorbed streptavidin coated beads, resolved in SDS-PAGE and detected by Western blot procedure for RelA/p65.

2.5. Western blots

Vero cells were infected and treated as described. At indicated times post infection, the cells were washed and scraped into PBS, centrifuged at 600 × *g* for 5 min and resuspended in RIPA lysis buffer (50 mM HEPES pH 7.5, 1% Igepal CA630, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 and 1:100 protease inhibitor cocktail). Following 15 min incubation on ice, samples were cleared by centrifugation at 14,000 × *g* for 15 min and 20 or 30 μg of heat-denatured protein was electrophoresed in 10% SDS-PAGE. Protein was transferred to nitrocellulose by semi-dry transfer and immunodetected by chemiluminescence (Amersham ECL Plus, UK).

2.6. Immunohistochemistry

Vero cells (4.0×10^4) were seeded into each chamber of 8-chambered slides (TissueTeck) and cultured for 24 h. Cells were mock or HSV-1 infected (moi = 10) and treated with resveratrol as described. At 6 h post infection, cells were washed with ice cold PBS, fixed with ice cold methanol for 5 min and air-dried for 8 min. Following two PBS washes, primary antibody (anti-RelA/p65 diluted 1:50 in 1.5% normal goat serum) was added and incubated overnight at 4 °C in a humid atmosphere. Slides were washed twice with PBS and then secondary antibody (HRP conjugated anti-rabbit diluted 1:400 in 1.5% normal goat serum) was added and incubated at room temperature for 1 h. The slides were washed twice with PBS and peroxidase activity detected with DAB (Sigma–Aldrich). Following development of substrate, the slides were washed with PBS, dehydrated with ethanol and xylene and cover-slipped using Permount.

2.7. Real-time RT-PCR

Vero cells were mock infected or infected with HSV-1 (moi = 5). At indicated times post infection, RNA was isolated and purified by the Trizol method (Invitrogen, Carlsbad, CA). RNA (1 μg) was subjected to DNase1 digestion (Sigma–Aldrich AMP-D1) as per manufacturer's protocol. cDNA was synthesized by first annealing the RNA with random hexamer primers (Promega, Madison, WI), in the presence of 0.5 mM dNTP mix. Next, the mixture was incubated with 200 units of MMLV-reverse transcriptase (Sigma–Aldrich M1302) and 20 units of RNAse inhibitor for 1 h at 37 °C. The MMLV-reverse transcriptase was heat denatured at 90 °C for 10 min and the samples stored at −20 °C until use. Total cDNA (50 ng) was added to SYBR Green Ready Start TaqPolymerase mixture (Sigma–Aldrich) with 100 nM each of forward and reverse primers for *ICP4* (2.5 mM $MgCl_2$) and *tk* (3.5 mM $MgCl_2$) (Kramer and Coen, 1995), *ICP0* (3 mM $MgCl_2$) (Cook et al., 2004), *ICP8* (3 mM $MgCl_2$) (Pesola et al., 2005), *HSV DNA pol* (3 mM $MgCl_2$) (Kubat et al., 2004), *glycoprotein C* (2.5 mM $MgCl_2$) forward 5′-GCGGCCAACCATCACCATGGAA-3′ and reverse 5′-AACCAGGCAAACGTCACGCCCT-3′ or *18s RNA* (1.5 mM $MgCl_2$) forward 5′-AGTCCCTGCCCTTTGTACACA-3′ and reverse 5′-GATCCGAGGGCCTCACTAAAC-3′. Samples were analyzed on an ABI 7700 sequencer (Applied Biosystems, Foster City, CA) with parameters of 94 °C 2 min polymerase activation phase, followed by 35–45 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C (*ICP0*), 52 °C (*ICP8* and *DNA pol*), 55 °C (*tk*), 58 °C (*18 s* and *gC*) and 60 °C (*ICP4*) for 30 s and elongation at 72 °C for 1 min. Primer efficiencies were validated using 10-fold serial dilutions of templates and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was employed to calculate the mean fold change in transcripts, using 18 s as an internal control. To verify the specificity of primers and absence of interfering HSV genomic DNA, negative controls included mock infection, no template and no reverse transcriptase.

2.8. Viral DNA synthesis

Vero cells (3.0×10^6) were seeded in 75 cm^2 flasks and cultured 24 h. The cells were infected with HSV-1 at an order of infection of 2.5 pfu per cell and treated with or without drug as described above. Cells were washed and harvested at 0 and 12 h post infection and frozen at −80 °C. Cell pellets of $\sim 2.0 \times 10^6$ cells were lysed in 300 μl of genomic DNA extraction buffer (0.2 M Tris–HCl [pH 8.5], 100 mM EDTA, 100 mM NaCl, 0.5% NP-40, 1% SDS and 100 μg/ml Proteinase K) for 3 h at 50 °C. DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 100% ethanol, washed with 70% ethanol and rehydrated in TE buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA). One nanogram of total DNA from each sample was added to SYBR Green Ready Start TaqPolymerase mixture with 100 nM each of forward and reverse primers for *tk* (Kramer and Coen, 1995) and analyzed using the parameters for real-time PCR described above. Viral DNA copies were calculated using a standard curve generated by purified HSV-1 DNA (Robbins et al., 1988). Briefly, confluent Vero cells in T175 cm^2 were infected with HSV-1 (moi = 10) and cultured for 16 h. Infected cells were harvested and lysed in LCM buffer (0.5% NP-40, 30 mM Tris–HCl [pH 7.5], 3.6 mM $CaCl_2$, 5 mM $MgCl_2$, 125 mM KCl, 0.5 mM EDTA and 6 mM 2-mercaptoethanol). The lipid envelope was extracted twice with Freon (1,1,2-trichloro-1,2,2-trigluoroethane) and viral capsids were pelleted from the aqueous phase using a glycerol step gradient (5 and 45% glycerol in LCM buffer) at 74,168 × *g* (Beckman L7/SW 28 ultra speed rotor, 1.25 h, 4 °C). The capsids were resuspended in TNE buffer (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 10 mM EDTA) and SDS was added to 0.5%. Phenol was added and the aqueous phase was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). The DNA was precipitated overnight in ethanol at −20 °C, pelleted at 16,319 × *g* (Sorvall SS34 rotor, 15 min, 4 °C) and resuspended in TE buffer. Viral DNA was quantified by spectrometry and diluted in series for each PCR run. The *tk* primer set and real-time PCR system allowed for detection of 10^2 to 10^7 copies of HSV-1 DNA, as determined by ABI Sequence Detector Software v.1.7.

2.9. Caspase-3 activity

Vero cells were infected and treated as described above. At various times post infection, 1×10^6 to 2×10^6 cells were scraped into media, centrifuged, and washed twice with ice cold PBS. Cell pellets were lysed in cell lysis buffer (20 mM Tris pH 7.2, 150 mM NaCl, 1% TX-100 and 1 mM DTT) by three freeze–thaw cycles. Samples were centrifuged at $16{,}000 \times g$ for 15 min at 4 °C, supernatants collected and analyzed for protein concentration by BCA protocol (Pierce). Cell lysates were then incubated at 37 °C for 4 h with a caspase-3 specific peptide substrate DEVD-pNA (Sigma–Aldrich) in caspase assay buffer (312.5 mM HEPES pH 7.5, 31.25%, w/v, sucrose and 0.3125%, w/v, CHAPS). Liberation of chromogenic *p*-nitroaniline (Abs = 405 nm) was measured in triplicate in 96-well plates. Data are reported as specific activity = [pmol pNA liberated/(hour × μg protein)] based on a standard curve generated from two-fold serial dilutions of pNA.

3. Results

3.1. Resveratrol suppresses HSV-1 induced activation of NF-κB

Electromobility shift assay was used to examine the effects of resveratrol on NF-κB in cells infected with HSV. Nuclear extracts were prepared from HSV infected Vero cells treated or not treated with resveratrol as described in Section 2. Fig. 1a demonstrates that HSV-1 at an moi of 10 stimulated activation of NF-κB. Maximal activation was seen at 6 h post infection and continued up to 24 h post infection, the end point of this study. However, in the presence of 219 μM resveratrol, NF-κB activation was suppressed in the infected cells. Band densitometry data at 24 h post infection (the point of maximal resveratrol induced NF-κB suppression) reveals a two-fold decrease of active NF-κB in HSV-1 infected cells treated with resveratrol (Fig. 1b). Similar results were obtained using an moi of 1 (data not shown).

To verify that the EMSA was specific to NF-κB activity, a biotin–streptavidin pull-down assay was performed. The results showed that the RelA/p65 component of NF-κB specifically bound the biotinylated κB response element because it was captured by the streptavidin beads and pulled-out of the nuclear extracts (Fig. 1c). A non-specific biotin probe was negative for RelA/p65 protein and a non-biotinylated competitor in 200:1 molar excess blocked RelA/p65 precipitation using the beads. Thus, the shifted bands in the EMSAs reflect at least one NF-κB protein, RelA/p65.

3.2. Resveratrol suppression of HSV-1 induced NF-κB activation is reversible

The reversibility of NF-κB inactivation by resveratrol was examined because previous studies showed that inhibition of HSV replication by resveratrol was reversible, whereby the removal of drug up to 48 h post infection allowed virus replication to resume (Docherty et al., 1999). Resveratrol (219 μM) was applied to mock infected or HSV-1 infected (moi = 10) cells for the first 12 h of infection. The cell lawns were washed with non-drug containing media and cultured for an additional 12 h in the absence of resveratrol. The results observed in the EMSAs demonstrate that when resveratrol treatment is removed after 12 h of infection, HSV-1 infected cells show greater activation of NF-κB compared to mock infected cells (Fig. 1a, reversal). These data agree with the previous findings about reversibility and demonstrate that resveratrol must be present in the media to exert suppression on NF-κB and inhibition of HSV replication.

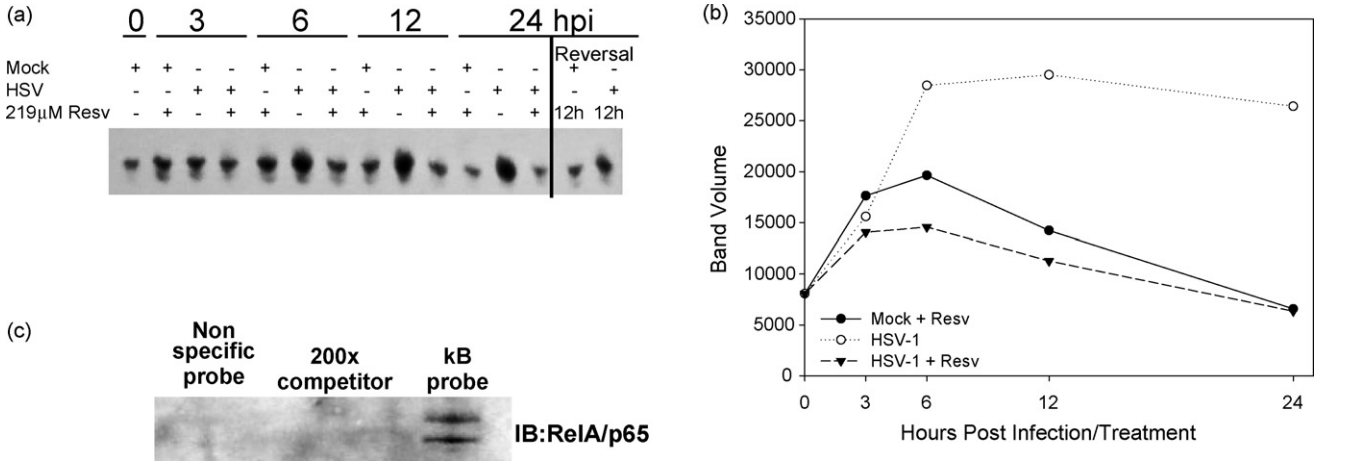

Fig. 1. Electromobility shift assays for NF-κB in HSV-1 infected Vero cells treated with resveratrol. (a) HSV-1 (moi = 10) infected Vero cells were examined for shifted bands in the presence or absence of 219 μM resveratrol from 0 to 24 h post infection. Lanes indicated by reversal represent samples treated with 219 μM resveratrol for 12 h, washed and incubated an additional 12 h in drug-free media. (b) Densities of shifted bands for mock infected with resveratrol (closed circle), HSV-1 infected (open circle) and HSV-1 infected with resveratrol (closed triangle). (c) Biotin–streptavidin pull-down assay. Nuclear extracts from HSV-1 infected cells were incubated with a non-specific biotinylated DNA probe (lane 1), the biotinylated κB probe and the non-biotinylated κB probe (competitor) in 200:1 molar excess (lane 2) or the biotinylated κB probe (lane 3). DNA–protein complexes were purified with streptavidin coated beads, electrophoresed in denaturing polyacrylamide and detected by Western blot for RelA/p65.

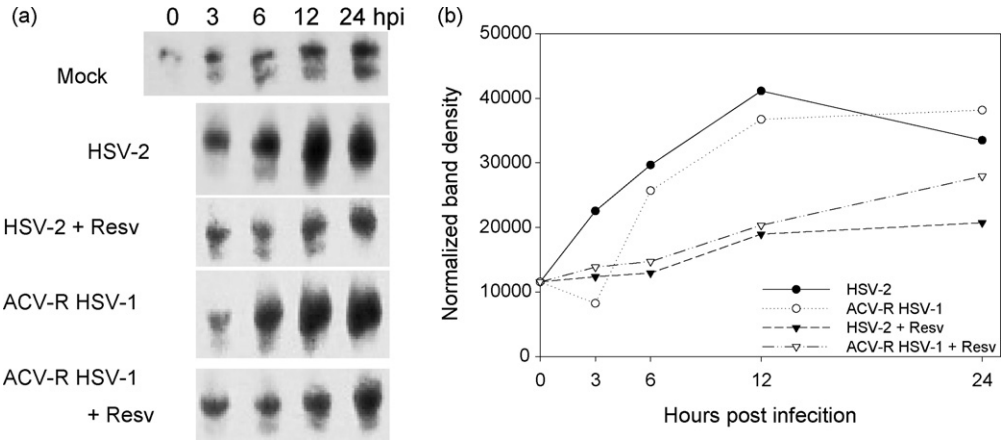

Fig. 2. Electromobility shift assays for NF-κB in HSV-2 and ACV-R HSV-1 infected Vero cells treated with resveratrol. (a) HSV-2 (moi = 10) and ACV-R HSV-1 (moi = 10) infected cells were examined for shifted bands in the presence or absence of 219 μM resveratrol from 0 to 24 h post infection. (b) Band densitometry data of shifted bands for HSV-2 (closed circle), ACV-R HSV-1 infected (open circle), HSV-2 infected with resveratrol (closed triangle) and ACV-R HSV-1 infected with resveratrol (open triangle).

3.3. Resveratrol suppresses NF-κB in HSV-2 and ACV-R HSV-1 infected cells

The previous experiment showed that resveratrol reduced activation of NF-κB in HSV-1 infected cells. These studies were repeated using cells infected with HSV-2 or ACV-R HSV-1. Both HSV-2 and ACV-R HSV-1 activated NF-κB (Fig. 2a). Maximal activation was approximately 12 h post infection for both virus types (Fig. 2). In the presence of resveratrol, NF-κB was suppressed in HSV-2 and ACV-R HSV-1 infected cells (Fig. 2). Band densitometry data, 12 h post infection, showed that resveratrol treatment decreased NF-κB by 53.9 and 44.7% in HSV-2 and ACV-R HSV-1 infected cells, respectively (Fig. 2b).

3.4. Resveratrol suppresses NF-κB activation in HSV-1 infected cells in a dose dependent manner

Electromobility shift assays were conducted to examine the effects of varying concentrations of resveratrol on NF-κB activation in HSV-1 infected cells. Previous studies showed that resveratrol affected HSV replication in a dose dependent fashion with 219 μM resveratrol reducing HSV titers by 99.9% (Docherty et al., 1999). Nuclear extracts from Vero cells infected with HSV-1 (moi = 1) were assayed for NF-κB activity 12 h post infection. Both 110 and 219 μM resveratrol inhibited NF-κB activity (Fig. 3). However, 219 μM resveratrol, the most effective anti-viral concentration, showed the greatest inhibitory effects on NF-κB, yielding 81.5% less active nuclear NF-κB than untreated, infected control (Fig. 3). These experiments were repeated using ACV-R HSV-1 and the same results were obtained (data not shown). These data demonstrate a parallel dose response of resveratrol inhibition of HSV replication and NF-κB suppression.

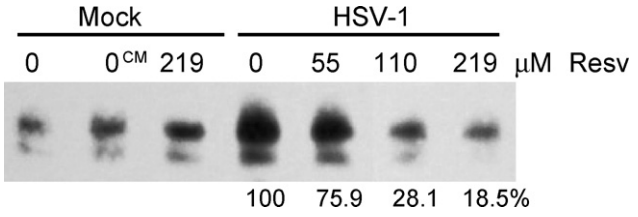

Fig. 3. Resveratrol blocks NF-κB activation in HSV-1 infected cells in a dose dependent manner. EMSAs were performed for NF-κB in Vero cells mock infected or infected with HSV-1 (moi = 1) and treated with 0, 55, 110 and 219 μM resveratrol or control media (0^{CM}). Values below lanes represent the percent of band density compared to HSV-1 infected, untreated control.

3.5. Resveratrol does not block nuclear translocation of NF-κB in HSV-1 infected cells

One of the critical steps in the NF-κB activation pathway is phosphorylation, dissociation and degradation of IκBα, the cytoplasmic inhibitory binding partner of NF-κB. Western blot analysis was used to determine the effects on IκBα during virus infection in the presence or absence of resveratrol. The occurrence of IκBα suggests that NF-κB is sequestered in the cytoplasm because IκBα is bound and masking the nuclear localization signal (nls) for NF-κB. Conversely, if IκBα is absent, then the nls is exposed and NF-κB is free to translocate to the nucleus. The results showed that mock infected cells not treated or treated with resveratrol maintained a relatively steady-state level of IκBα. However, HSV-1 infected cells showed disappearance of IκBα by 12 h post infection (Fig. 4a). Similarly, the addition of resveratrol to HSV-1 infected cells showed degradation of IκBα by 12 h post infection (Fig. 4a). These results suggest that resveratrol did not protect IκBα from HSV-1 associated degradation. These experiments were also repeated using ACV-R HSV-1 and the same results were obtained (data not shown).

Next, immunohistochemistry for RelA/p65, a component of NF-κB, was employed 6 h post infection to assess localization within the cell. In mock infected cells treated with control media or 219 μM resveratrol the cytoplasm stained evenly for NF-κB and the nuclei showed little to no positive staining (Fig. 4b), indicating retention of NF-κB in the cytoplasm. However, HSV-1 infected cells treated with control media showed translocation of NF-κB to the nucleus, as evidenced by dark staining for both

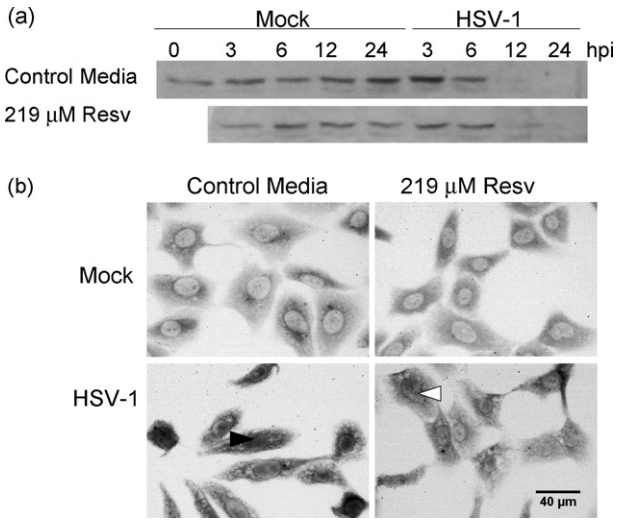

Fig. 4. Resveratrol does not block nuclear translocation of NF-κB in HSV-1 infected cells. (a) Western blot analysis for IκBα in mock infected or HSV-1 (moi = 1) infected Vero cells in the presence or absence of 219 μM resveratrol. (b) Immunohistochemistry for detection of RelA/p65, an NF-κB protein, in Vero cells 6 h post infection with HSV-1 (moi = 10) and treatment with control media or 219 μM resveratrol.

cytoplasm and nuclei (Fig. 4b, closed arrow). Resveratrol treatment does not alter this staining pattern (Fig. 4b, open arrow). These tests indicate that RelA/p65 translocates to the nucleus of HSV-1 infected cells as early as 6 h post infection, even in the presence of the resveratrol.

3.6. Resveratrol does not induce apoptosis in HSV-1 infected cells

Previous reports illustrated that NF-κB induction in HSV-1 infected cells is anti-apoptotic (Goodkin et al., 2003; Gregory et al., 2004). To determine if the loss of NF-κB activity in HSV-1 infected cells treated with resveratrol results in apoptosis, caspase-3 activity assays were performed. The data revealed that resveratrol induced caspase-3 activity in mock infected cells (Fig. 5a). However, infection with HSV-1 blocked caspase-3 activation in the presence or absence or 219 μM resveratrol (Fig. 5a). Therefore, caspase-3 induced apoptosis is prevented in HSV-1 infected cells treated with resveratrol.

The observation that resveratrol alone may induce caspase-3 activation was unexpected. Vero cell cultures are 91.5 and 72.0% viable at 48 and 72 h post resveratrol treatment, as determined by MTT cytotoxicity and trypan blue exclusion assays (data not shown). Additionally, Vero cells treated with 219 μM resveratrol show evidence of cell cycle arrest, not apoptosis, via flow cytometry (Docherty et al., 1999) and induction of a cyclin dependent kinase inhibitor, p21 (data not shown). Thus, a reversibility experiment was designed to further test the induction of caspase-3 activity. Vero cells were incubated with 219 μM resveratrol for 24 or 48 h, then the drug containing media was removed, the cells washed and non-drug containing media applied for the remainder of the experiment. When resveratrol treatment is removed after 24 and 48 h, caspase-3 activity returned to basal levels (Fig. 5b). This observation in conjunction with the viability data suggests that the caspase-3 activity threshold for inducing apoptosis is not reached by 219 μM resveratrol treatment within the time limit of the experiment.

3.7. Immediate-early, early and late HSV-1 gene expression and viral DNA synthesis

HSV gene activation and DNA synthesis were studied to identify the period of the viral life cycle affected by resveratrol treatment. Real-time RT-PCR assays were used to determine the HSV mRNA content in infected cells with or without resvera-

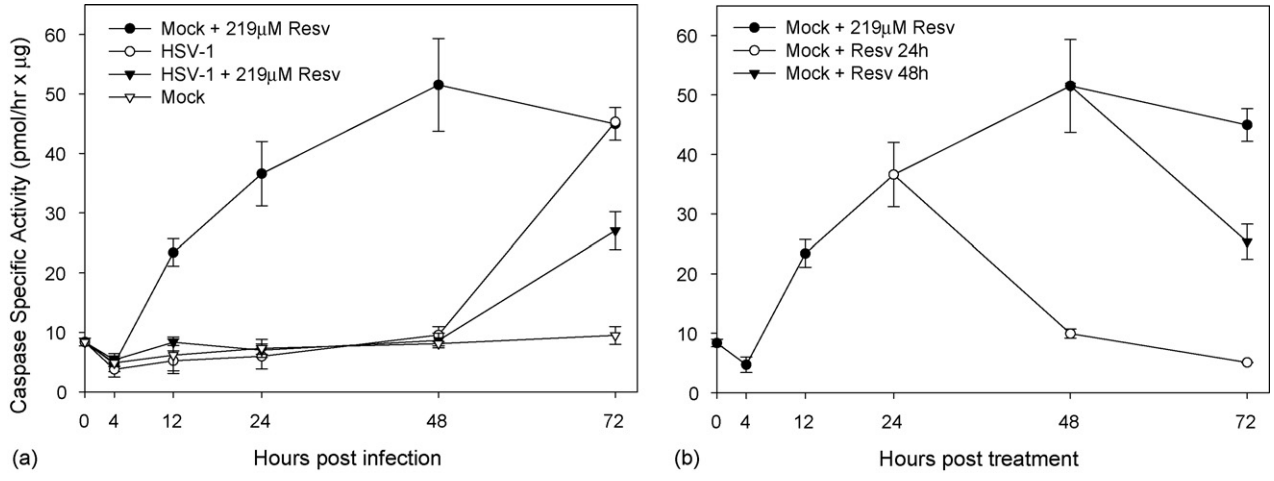

Fig. 5. Caspase-3 activity in HSV-1 infected cells treated with resveratrol. Vero cells were mock or HSV-1 infected (moi = 1) and cultured in the presence or absence of 219 μM resveratrol. At the designated times post infection cell lysates were collected and incubated with a caspase-3 specific peptide, DEVD-pNA. Absorbance at 405 nm was used to detect pNA. The data are reported as the mean (±S.D.) of triplicate samples analyzed for the specific activity = pmol of liberated pNA per hour per μg lysate. (a) Vero cells were mock infected and treated with 219 μM resveratrol (closed circle), infected with HSV-1 (open circle), HSV-1 infected and treated with 219 μM resveratrol (closed triangle) or mock infected (open triangle). (b) Vero cells were tested for the reversibility of caspase-3 activity by treatment with 219 μM resveratrol for 72 h (closed circle), 219 μM resveratrol for 24 h then no drug 48 h (open circle) and 219 μM resveratrol for 48 h then no drug 24 h (closed triangle).

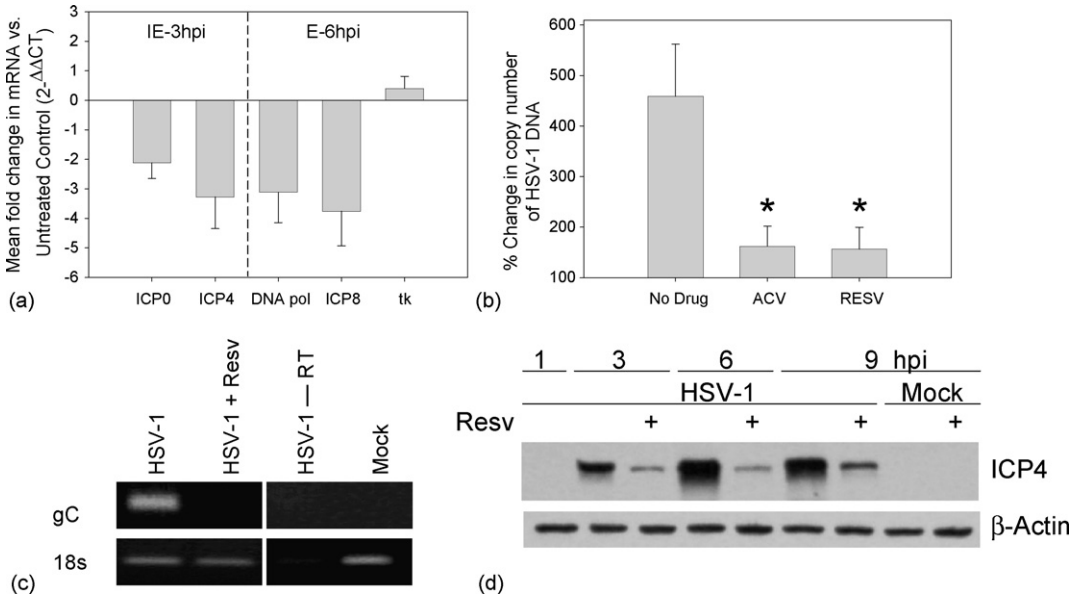

Fig. 6. Immediate-early, early and late HSV-1 gene expression and viral DNA synthesis. (a) Real-time RT-PCR was used to determine the mean fold change of ICP4 and ICP0 (3 hpi), HSV DNA polymerase (DNA pol), ICP8 and *tk* (6 hpi) mRNA in Vero cells treated with 219 μM resveratrol. The reported data were calculated using the $2^{-\Delta\Delta Ct}$ method and represents the mean (±S.D.) of two separate experiments analyzed in triplicate. (b) Real-time PCR was employed to calculate the change in HSV-1 genome copy number at 12 h post infection in samples treated without drug, 10 μM acyclovir (ACV) or 219 μM resveratrol. Data were analyzed using independent *t*-tests comparing untreated samples to either ACV or resveratrol treatment, $^{*}p < 0.001$. (c) Detection of mRNA for glycoprotein C (gC) at 6 hpi in the absence or presence of 219 μM resveratrol. Bands represent the PCR products following 40 cycles of real-time RT-PCR electrophoresed in 1.5% agarose and visualized with ethidium bromide. (d) Western blot analysis for ICP4 in the presence or absence of 219 μM resveratrol.

trol treatment. First mRNA for two immediate-early genes, ICP0 and ICP4, were assayed at 3 h post infection. The results showed that mRNA for ICP0 is reduced 2.1-fold and mRNA for ICP4 is reduced 3.3-fold when resveratrol is present at 219 μM (Fig. 6a). To complement these studies, Western blot analysis for ICP4 was conducted to validate reductions of mRNA observed by real-time PCR analysis. The data show that at 3 h post infection, ICP4 was minimally detected if resveratrol was present (Fig. 6d). At 9 h post infection, there was a slight increase in ICP4 protein in the presence of resveratrol, but it was still significantly less than the ICP4 found at 9 h post infection in untreated samples. Thus, resveratrol treatment is interfering with activation of the first kinetic class of HSV genes.

Next, early viral gene activation, represented by the HSV DNA polymerase (pol), single-stranded DNA binding protein (ICP8) and thymidine kinase (*tk*), was analyzed by real-time RT-PCR at 6 h post infection. The results in Fig. 6a demonstrated that mRNA for pol and ICP8 were reduced 3.1- and 3.8-fold, respectively. However, there was not a significant negative effect on *tk* mRNA by resveratrol. These data suggest that some early genes are also affected by resveratrol treatment.

The next phase of viral replication, DNA synthesis was evaluated. Using quantitative PCR, the copy number of HSV DNA was determined in untreated cells and cells treated with a known HSV DNA synthesis inhibitor, acyclovir (ACV), or 219 μM resveratrol. The data show that resveratrol treatment inhibited HSV DNA synthesis similar to ACV treatment (Fig. 6b). Additionally, the difference in HSV DNA copy number for resveratrol or ACV treatment was significantly less than untreated samples, $p < 0.001$. This observation may reflect the observed reductions of essential early genes required for HSV DNA synthesis.

Lastly, late gene activation, which is principally dependent on DNA synthesis, was evaluated by assaying for glycoprotein C (gC) mRNA. Initially, this experiment was performed using the real-time RT-PCR procedure. However, no product was produced and could not be calculated using the $2^{-\Delta\Delta Ct}$ method. Thus, the end products following 40 cycles of PCR were electrophoresed in agarose and visualized with ethidium bromide. Fig. 6c shows that treatment with resveratrol reduced gC mRNA to undetectable levels at 6 h post infection. These studies demonstrate that resveratrol negatively affects mRNA synthesis for two immediate-early and two early genes, leading to significant inhibition of DNA synthesis and late gene activation. Collectively, resveratrol shows inhibition at the earliest phases of HSV replication, compounding to greater effects later in the viral life cycle.

4. Discussion

Resveratrol has been shown to inhibit HSV-1 and HSV-2 in vitro (Docherty et al., 1999) and in vivo (Docherty et al., 2004, 2005), but the mechanism(s) by which this occurs is uncertain. The studies presented here were designed to examine the possible mechanism(s) by which resveratrol inhibits HSV. Electromobility shift assays demonstrated that HSV-1, HSV-2 and acyclovir resistant HSV-1 activate NF-κB and that this activation is reduced by resveratrol. The capacity of resveratrol to suppress NF-κB activation parallels its anti-herpetic effects reported by Docherty et al. (1999) when tested over a range of concentrations. Furthermore, resveratrol exerts its suppressive

effects on NF-κB within the nucleus, given that RelA/p65 translocation to the nucleus of HSV infected cells is not blocked by resveratrol. Resveratrol demonstrated direct effects on HSV-1 gene activity by reducing synthesis of mRNA for ICP0, ICP4, ICP8 and HSV DNA polymerase and preventing synthesis of mRNA for glycoprotein C. Furthermore, resveratrol treated cells do not allow for HSV DNA synthesis, a critical phase of the viral life cycle, to proceed.

HSV activation of NF-κB was previously described (Patel et al., 1998; Amici et al., 2001, 2006; Goodkin et al., 2003; Gregory et al., 2004), as was its activation by other members of the herpes virus family including HHV-8 (Liu et al., 2002), human cytomegalovirus (HCMV) (DeMeritt et al., 2004) and Epstein-Barr virus (EBV) (Sugano et al., 1997). Resveratrol was found to block NF-κB activation in HCMV infected cells and block synthesis of immediate-early viral proteins IE1-72 and IE2-86 (Evers et al., 2004). Congruent to the aforementioned study, we observe that the expression of two immediate-early viral genes in HSV-1 infected cells, ICP0 and ICP4, are reduced in the presence of resveratrol. Even though there are numerous reports indicating that herpes viruses activate NF-κB, the viral requirement for this cellular factor still remains elusive. NF-κB may be activated to control the host cell for productive viral replication or utilized directly by herpes viruses to control transcription. One study using HEp-2 cells reports that NF-κB has anti-apoptotic effects during HSV-1 infection (Goodkin et al., 2003). Another cites that NF-κB activation depended on another cellular factor, protein kinase R, yet plays no role in apoptosis prevention in SK-N-SH cells (Taddeo et al., 2003). Since NF-κB activity may modulate the apoptotic responses in HSV infected cells (Goodkin et al., 2003; Gregory et al., 2004) and ICP4 deficient HSV mutants were found to initiate apoptosis (Leopardi and Roizman, 1996; Nguyen et al., 2005), we inspected apoptosis by caspase-3 activity in HSV infected cells treated with 219 μM resveratrol. We found no evidence that resveratrol induces caspase-3 mediated apoptosis in HSV-1 infected Vero cells. In terms of HSV utilizing NF-κB directly, it was recently reported that NF-κB is recruited to the promoter of ICP0 in HSV-1 infected keratinocytes (Amici et al., 2006). We show that ICP0 mRNA is reduced by two-fold in the presence of resveratrol. If NF-κB is utilized by the virus to activate ICP0 gene expression, then the two-fold reduction may reflect NF-κB suppression at a very early time after infection even though the EMSAs do not demonstrate significant differences during this period. Thus, the anti-herpetic effects achieved by resveratrol may reside in its inhibitory effects on NF-κB unrelated to the abortive host process of apoptosis.

The first steps to characterize the mechanism of inhibition of NF-κB in HSV infected cells were to examine the cytoplasmic pathways that lead to NF-κB activation. Resveratrol and other polyphenols were reported to inhibit the phosphorylation and subsequent degradation of IκBα within the cytoplasm, a step preceding the translocation of NF-κB to the nucleus (Homles-McNary and Baldwin, 2000; Ashikawa et al., 2002). Thus, IκBα was probed by Western blot to elicit an activation pathway both utilized by HSV and blocked by drug treatment. The data showed that HSV-1 infection rapidly degrades IκBα, even in the presence of resveratrol, suggesting nuclear localization of NF-κB. To determine if NF-κB would translocate to the nucleus in the absence of IκBα, immunohistochemisty for RelA/p65, a component of NF-κB was performed. These experiments suggested that NF-κB localized to the nucleus during HSV-1 infection and that resveratrol treatment did not prevent this translocation. Analogous data were reported in a study using human umbilical cord endothelial cells that determined that prior to 24 h post treatment, resveratrol did not inhibit translocation of NF-κB when TNF-alpha was the agonist (Pellegatta et al., 2003). Thus, resveratrol can also block the activity of NF-κB once it enters the nucleus.

Lastly, the direct effects of resveratrol on the HSV life cycle are critical to understanding the link of NF-κB suppression to the inhibition of virus replication. Resveratrol interfered with the expression of immediate-early and early gene activation with the exception of thymidine kinase. However, the decrease of ICP4 mRNA and protein yielding no change of thymidine kinase mRNA is not unfounded. ICP4 mutants of HSV-1 have been shown to produce mRNA for *tk* by relying on transcriptional factors other than ICP4 for activation (Samaniego et al., 1995; Wu et al., 1996). Yet, two other early genes, ICP8 and pol, show reductions in mRNA reflecting the reductions of ICP4 and possibly other immediate-early genes. These reductions lead to the inhibition of subsequent phases of HSV replication, DNA synthesis and late gene activation. Interestingly, it was demonstrated previously that resveratrol was ineffective at blocking HSV replication when added after 6 h post infection in vitro (Docherty et al., 1999). At 6 h post infection, transcripts for immediate-early and early genes have been synthesized and the viral DNA is being synthesized. When comparing this time point to the activation and suppression of NF-κB in our system it is observed that maximal activation for HSV-1 is also at 6 h post infection. These data do not clearly differentiate which inhibition event occurs first and it is not evident whether the reductions in NF-κB by resveratrol impede virus replication or whether the reductions in viral products by resveratrol lead to less activation of NF-κB. However, the translocation studies show that infection with HSV-1 leads to NF-κB translocation to the nucleus of both untreated and resveratrol treated cells by 6 h post infection. At least one step of the activation of NF-κB will proceed in both cases. It is the presence of the chemical that suppresses activity of NF-κB once inside the nucleus. Therefore, we propose that resveratrol has a separate mechanism to interfere with nuclear NF-κB activity in HSV infected cells.

Cumulatively, the data presented in this report describe how resveratrol negatively alters a host factor, NF-κB, in HSV infected cells resulting in inhibition of virus replication, the impairment of activation of essential immediate-early, early and late genes and the inhibition of DNA synthesis. While these findings of NF-κB suppression by resveratrol in HSV infected cells could explain in part the anti-herpetic activities of the drug, we do not exclude the possibility of involvement of other cell factors in this mechanism. Resveratrol has proven to affect multiple targets within cells, as reviewed by Aggarwal et al. (2004). Thus, further research of these constituents may help to characterize additional cell factors utilized by herpes viruses, advancing the

knowledge of herpes virus replication and discovering novel cellular targets for anti-viral drug development.

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