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Activation of IκB Kinase by Herpes Simplex Virus Type 1

A NOVEL TARGET FOR ANTI-HERPETIC THERAPY*

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Herpes simplex viruses (HSV) are ubiquitous pathogens causing a variety of diseases ranging from mild illness to severe life-threatening infections. HSV utilize cellular signaling pathways and transcription factors to promote their replication. Here we report that HSV type 1 (HSV-1) induces persistent activation of transcription factor NF-κB, a critical regulator of genes involved in inflammation, by activating the IκB kinase (IKK) in the early phase of infection. Activated NF-κB enhances HSV-1 gene expression. HSV-1-induced NF-κB activation is dependent on viral early protein synthesis and is not blocked by the anti-herpetic drug acyclovir. IKK inhibition by the anti-inflammatory cyclopentenone prostaglandin A_1 blocks HSV-1 gene expression and reduces virus yield by more than 3000-fold. The results identify IKK as a potential target for anti-herpetic drugs and suggest that cyclopentenone prostaglandins or their derivatives could be used in the treatment of HSV infection.

One intriguing aspect of herpesviruses is their ability to influence host defense mechanisms and replication of other pathogens by inducing a stress response, via activation of cellular transcription factors, among which is nuclear factor-κB (NF-κB).[1] NF-κB is a critical regulator of the immediate-early pathogen response, playing an important role in promoting inflammation and viral gene expression (1). In most eukaryotic cells NF-κB exists as an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65 (Rel A) subunits, bound to inhibitory proteins of the IκB family, usually IκBα (1, 2). NF-κB is activated in response to a variety of stress and pathogenic stimuli, including UV radiation, bacterial and viral infection, and proinflammatory cytokines (2, 3). Several stimuli activate NF-κB by augmenting the activity of the IκB kinase (IKK) complex, containing two catalytic subunits (IKK-α and IKK-β) and the IKK-γ or NEMO regulatory subunit (4, 5). IKK phosphorylates IκBs at sites that trigger their ubiquitination and proteasome-mediated degradation (1, 4). Freed NF-κB dimers translocate to the nucleus and activate a variety of genes encoding adhesion molecules, inflammatory and chemotactic cytokines, cytokine receptors, and enzymes that produce inflammatory mediators (1, 2). NF-κB also activates the transcription of viral genes and is involved in several pathological events including progression of AIDS by enhancing human immunodeficiency virus type 1 (HIV-1) transcription (6, 7). Several viruses, including HIV-1 (8), cytomegalovirus (9), SV40 (10), and hepatitis B virus (11), contain functionally important NF-κB-binding sites. In the case of HSV-1, NF-κB-binding sites are located in the *ICP0* and *Vmw65* genes (12).

In addition to containing NF-κB-binding sites in its genome, HSV-1 can also induce NF-κB nuclear translocation (13), and two immediate-early (IE) proteins, ICP4 and ICP27, were found to be required for this effect (13, 14); however, the signaling pathway utilized by the virus to activate the factor has still not been defined.

We now report that, like proinflammatory cytokines, HSV-1 induces NF-κB by the activation of IKK, followed by IκBα degradation, in the early phase of infection. As we have recently shown that cyclopentenone prostaglandins (cyPG) of the A and J type, which possess anti-inflammatory (15) and antiviral (16) activity, block TNFα-induced NF-κB activation by direct inhibition and modification of the IKKβ subunit of IKK (17), we have investigated their effect on IKK and NF-κB activation during HSV-1 infection. We report that cyPG are powerful inhibitors of IKK and NF-κB activation induced by HSV-1 infection and that this effect results in a potent antiviral activity in human cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Treatments—Human HEp-2 laryngeal carcinoma cells and neuroblastoma SK-N-SH cells, and monkey VERO cells were grown at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Cell viability was determined by dye exclusion technique or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to MTT formazan conversion assay. Prostaglandins (Cayman Chemicals) dissolved in ethanol were used as described (18). MTT, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), tumor necrosis factor (TNFα), actinomycin D, cycloheximide, and acyclovir were obtained from Sigma. Transfections were carried out using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's protocols. Reporter plasmids pIE3-CAT, containing the HSV-1 ICP4 promoter (gift from Dr. R. D. Everett), and 2xNF-κB-LUC (gift from Dr. M. Karin), and IκBα super-repressor (IκBα-AA) expression vector have been described (4, 19). After transfection, cells were maintained in RPMI 1640 containing 10% fetal calf serum for 24 h before infection with HSV-1. Twelve h after virus infection, cell extracts were prepared for gene reporter assays as described (17, 18).

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[1] The abbreviations used are: NF-κB, nuclear factor-κB; HSV, herpes simplex viruses; HSV-1, HSV, type 1; IKK, IκB kinase; IE, immediate-early; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; JNK, c-Jun amino-terminal kinase; p.i., post-infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIV-1, human immunodeficiency virus type 1; pfu, plaque-forming units; cyPG, cyclopentenone prostaglandins; PG, prostaglandins; CAT, chloramphenicol acetyltransferase.

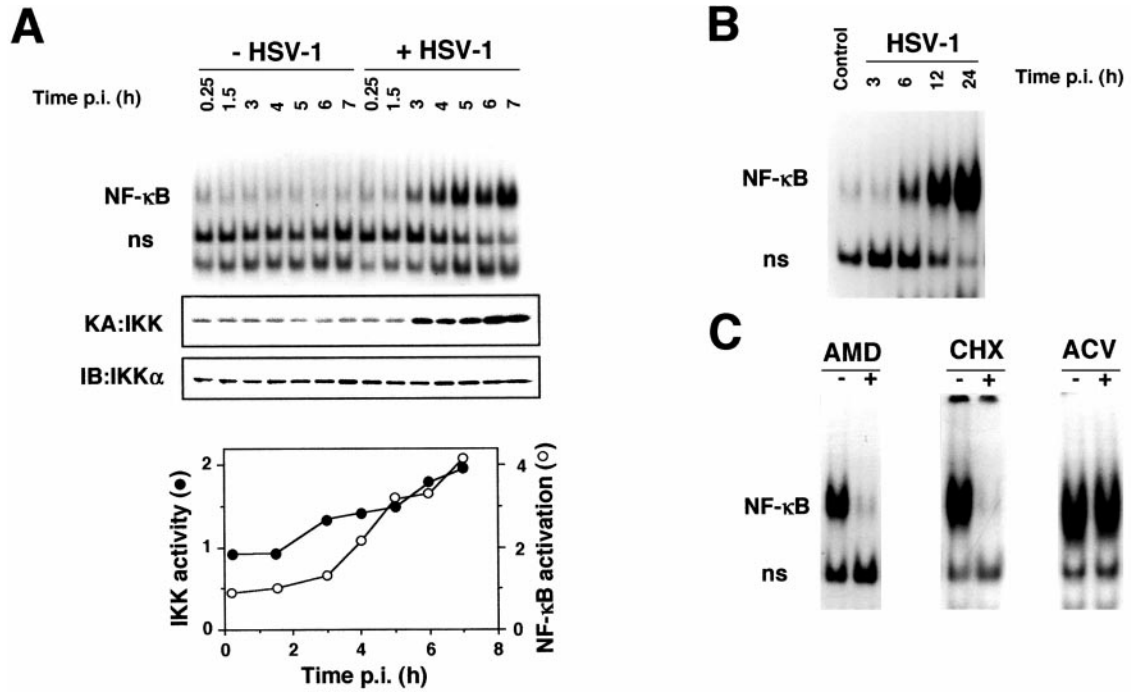

FIG. 1. **HSV-1 infection induces IKK and NF-κB activities in HEp-2 cells.** *A,* HEp-2 cells were infected with HSV-1 (10 pfu/cell) for 1 h at 37 °C. At different times p.i., whole-cell extracts from uninfected (*−HSV-1*) or HSV-1-infected (*+HSV-1*) cells were prepared and assayed for NF-κB activation by EMSA (*top panel*) and IKK activity by kinase assay (*KA*, *middle panel*). Positions of NF-κB-DNA (*NF-κB*) and a nonspecific protein-DNA (*ns*) complexes are indicated. Endogenous IKK recovery was determined by immunoblotting for IKKα (*IB:IKKα*). The levels of NF-κB DNA-binding (○) and IKK activity (●) in uninfected or HSV-1-infected HEp-2 cells were quantified by Molecular Dynamics PhosphorImager analysis and expressed as fold induction of the levels detected in uninfected control cells (*bottom panel*). *B,* HEp-2 cells were infected with HSV-1 (1 pfu/cell) for 1 h at 37 °C. At different times p.i., whole-cell extracts from uninfected (*Control*) or HSV-1-infected (*HSV-1*) cells were assayed for NF-κB activation by EMSA. *C,* HSV-1-infected HEp-2 cells were treated with actinomycin D (*AMD*, 5 μg/ml), cycloheximide (*CHX*, 50 μg/ml) or acyclovir (*ACV*, 5 μg/ml) soon after the 1-h adsorption period. Whole-cell extracts at 8 h p.i. were analyzed for activation of NF-κB by EMSA. Sections of fluorograms from native gels are shown.

Virus Infection—HEp-2 cell monolayers were infected for 1 h at 37 °C with HSV-1 strain F1 at a multiplicity of infection of 10 pfu/cell, unless stated otherwise. Prostaglandins were added after the 1-h adsorption period and maintained in the medium for the duration of the experiment. Controls received an equal amount of ethanol, which did not affect cell metabolism or virus replication. Virus titers were determined at different times post-infection (p.i.) by plaque assay or by cytopathic effect 50% ($CPE_{50\%}$) assay on confluent Vero cell monolayers (20). Statistical analysis was performed using the Student's *t* test for unpaired data. Data were expressed as the mean ± S.E., and *p* values of < 0.05 were considered significant.

Protein Synthesis and Western Blot Analysis—Cells were pulse-labeled with [^{35}S]methionine (10 μCi/10^5 cells) and lysed in L buffer (20 mM Tris-Cl, pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5% SDS). Samples containing the same amount of radioactivity were separated by SDS-PAGE (3% stacking gel, 10% resolving gel) and processed for autoradiography (18). For immunoblot analysis, equal amounts of protein (50 μg/sample) from HEp-2 whole-cell extracts were separated by SDS-PAGE, blotted to nitrocellulose, and filters were incubated with monoclonal anti-hsp70 antibodies (Amersham Pharmacia Biotech), polyclonal anti-IκBα/MAD3 (Santa Cruz Biotechnology), anti-IKKα or anti-JNK1 (PharMingen) antibodies followed by decoration with peroxidase-labeled anti-mouse or anti-rabbit IgG (ECL, Amersham Pharmacia Biotech) (17).

Electrophoretic Mobility Shift Assay (EMSA)—Whole-cell extracts were prepared after lysis in a high salt extraction buffer (18). Aliquots of total or nuclear extracts (15 or 5 μg of protein, respectively) were incubated with ^{32}P-labeled κB DNA probe (18) followed by analysis of DNA binding activities by EMSA. Binding reactions were performed as described (18). Complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis. Specificity of protein-DNA complexes was verified by immunoreactivity with polyclonal antibodies specific for p65 (Rel A). Quantitative evaluation of NF-κB-κB complex formation was determined by Molecular Dynamics PhosphorImager analysis.

Kinase Assay—Cell lysates were incubated with anti-IKKα or anti-JNK1 antibodies in the presence of 15 μl of protein-A-Sepharose at 4 °C for 12 h. After extensive washing, endogenous IKK and JNK1 activities were determined using GST-IκBα-(1–54) and GST-c-Jun-(1–79) as substrates, respectively (17). Western blot analysis was performed as a kinase loading control.

Northern Blot Analysis—Total RNA from uninfected and virus-infected cells was isolated by the guanidinium isothiocyanate method (21), fractionated (5 μg) on 1% agarose/formaldehyde gels, and transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech). For detection of HSV-1 ICP4 mRNA, filters were hybridized with ^{32}P-labeled pRB3094 probe (a gift from Dr. R. Monservigi) and processed for autoradiography. After being stripped, filters were rehybridized with an antisense oligonucleotide specific for the glyceraldehyde-phosphate dehydrogenase gene (GAPDH) 5′-end-labeled by T4 kinase with [γ-^{32}P]ATP (Amersham Pharmacia Biotech), as a loading control (21).

RESULTS

HSV-1 Infection Activates IKK and NF-κB in Human Cells—Confluent HEp-2 cell monolayers were infected with HSV-1 for 1 h at 37 °C. After the 1-h adsorption period, the virus inoculum was removed, and cells were incubated at 37 °C for the following 24 h. Mock-infected cells were treated identically. At different times p.i., whole-cell extracts were analyzed for NF-κB activation by EMSA and IKK activity by kinase assay. The level of NF-κB DNA binding activity and phosphorylated IκBα were quantitated by Molecular Dynamics PhosphorImager analysis. HSV-1 potently induced IKK and NF-κB activation in HEp-2 cells starting at 3 h p.i. (Fig. 1*A*). NF-κB activation was independent of the multiplicity of infection and persisted for at least 24 h (Fig. 1*B*).

The IE proteins ICP4 and ICP27 were found to be required for NF-κB nuclear translocation following infection of C33 cells with HSV-1 (13). To determine whether NF-κB activation in HEp-2 cells was dependent on *de novo* synthesized viral products, cell monolayers were infected with HSV-1 in the presence or in the absence of actinomycin D (5 μg/ml), cycloheximide (50 μg/ml), or the antiviral drug acyclovir (5 μg/ml). Eight hours

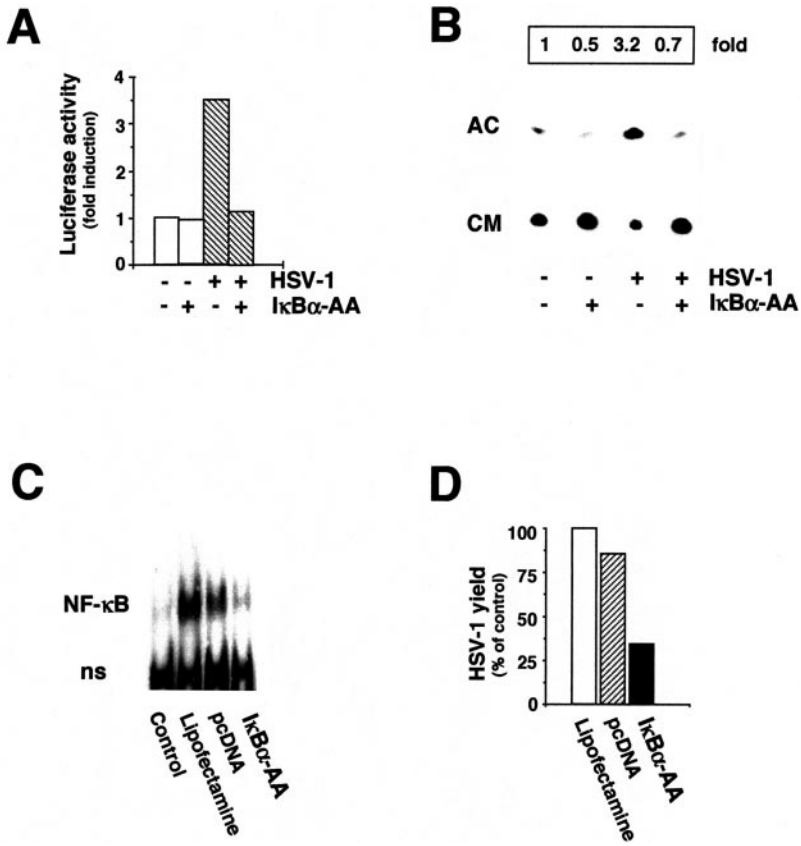

FIG. 2. **Effect of NF-κB activation on HSV-1 replication.** *A,* HEp-2 cells were co-transfected with a 2xNF-κB-LUC reporter, together with empty (−) or IκBα-AA (+) vectors. After 24 h, cells were infected with HSV-1 (+) or were mock-infected (−). Cells were assayed for luciferase activity 12 h p.i. in duplicate samples. The activity achieved in mock-infected cells in the absence of IκBα-AA was given an arbitrary value of 1, and the other values were calculated relative to that. *B,* HEp-2 cells were co-transfected with the IE3-CAT reporter together with empty (−) or IκBα-AA (+) vectors, and after 24 h were infected with HSV-1 (+) or mock-infected (−). CAT assay was performed 12 h p.i. in duplicate samples and quantified by Molecular Dynamics PhosphorImager analysis. The activity is expressed as fold increase of levels in mock-infected cells in the absence of IκBα-AA (*top panel*). *AC*, acetylated form of [^{14}C]-1-deoxychloramphenicol; *CM*, unreacted reagent. *C* and *D,* HEp-2 cells transfected with empty (*pcDNA*) or IκBα-AA (*IκBα-AA*) vectors were infected with HSV-1 (2 pfu/cell) or mock-infected (*Control*) 24 h after transfection. NF-κB DNA binding activity was determined by EMSA in whole-cell extracts 16 h after infection (*C*). At the same time, virus titers were determined by $CPE_{50\%}$ assay in the supernatants of transfected or mock-transfected (LipofectAMINE) cells (*D*). Data are expressed as percentage of virus yield in untreated mock-transfected cells and represent the average from two separate experiments.

p.i., whole-cell extracts were prepared and subjected to EMSA. As shown in Fig. 1*C*, HSV-1-induced NF-κB activation is dependent on protein synthesis, since it was inhibited by both actinomycin D or cycloheximide treatment. Acyclovir, which does not affect the synthesis of viral α and DNA replication-independent proteins, had no effect on NF-κB activation by HSV-1, indicating that the synthesis of immediate-early or early viral gene products is responsible for NF-κB induction in HEp-2 cells.

Role of NF-κB in HSV-1 Replication—The HSV-1 genome harbors several consensus binding sites for NF-κB (12). However, the importance of NF-κB activation and the potential involvement of κB sites in the progression of the virus replication cycle are not completely understood. We have then investigated the role of NF-κB on HSV-1 gene expression. To investigate whether HSV-1-induced NF-κB was transcriptionally active, we utilized the IκBα super-repressor IκBα-AA, in which $Ser^{32/36}$ residues critical for phosphorylation by IKK have been replaced by alanine (4). HEp-2 cells were transiently co-transfected with a 2xNF-κB-LUC reporter, together with "empty" or IκBα-AA vectors. After 24 h, cells were infected with HSV-1. Cells were lysed and assayed for luciferase activity 12 h p.i. HSV-1 infection induced luciferase expression, and this effect was prevented by co-transfection with IκBα-AA (Fig. 2*A*), indicating that HSV-1-induced NF-κB is transcriptionally functional. In a different experiment, HEp-2 cells were co-transfected with the IE3-CAT reporter, containing the HSV-1 ICP4 promoter, together with empty or IκBα-AA vectors, and after 24 h were infected with HSV-1. CAT assay was performed 12 h p.i. As expected, HSV-1 infection induced pIE3 expression (Fig. 2*B*). Co-transfection with IκBα-AA completely blocked pIE3 expression, indicating that NF-κB is involved in the regulation of HSV-1 IE gene transcription. In a different experiment HEp-2 cells transfected with IκBα-AA or empty vectors were infected with HSV-1 24 h after transfection. At 16 h p.i. NF-κB DNA-binding activity was determined by EMSA in the cellular extracts, and virus titers were determined by $CPE_{50\%}$ assay in the supernatant of infected cells. As shown in Fig. 2*C*, expression of IκBα-AA was effective in inhibiting HSV-1-induced NF-κB activation and resulted in a 70% reduction of virus yield in the supernatant of infected cells (Fig. 2*D*). These results indicate that transcriptionally active NF-κB plays an important role for efficient viral replication in HSV-1-infected human cells.

The effect of different NF-κB inducers on HSV-1 replication was also analyzed. HEp-2 cells were infected with HSV-1 and maintained at 37 °C in the presence or absence of TPA (25 ng/ml) or TNFα (10 ng/ml). Mock-infected cells were treated identically. Eight hours p.i., cells were harvested, and the levels of NF-κB DNA-binding activity were determined. HSV-1 infection induced NF-κB activation; levels of NF-κB DNA binding were found to be doubled in TPA-treated HSV-1-infected cells (Fig. 3*A*). In parallel samples HSV-1 ICP4 mRNA levels and infectious particle production were determined at 8 h p.i. by Northern blot analysis and by $CPE_{50\%}$ assay, respectively. Both ICP4 mRNA levels and virus titers were increased by more than 100% by TPA treatment (Fig. 3, *B* and *C*). Similar results were shown in TNFα-treated HSV-1-infected cells (data not shown), indicating that enhancement of NF-κB activation by different stimuli contributes to increase the efficiency of HSV-1 replication.

PGA_1 Prevents HSV-1-induced IKK and NF-κB Activation and Inhibits Virus Replication—We have recently shown that cyPG inhibit NF-κB induced by TPA or TNFα treatment in Jurkat lymphoblastoid cells by preventing IKK activation (17). To investigate whether cyPG interfere with virus-mediated induction of IKK and NF-κB, HEp-2 cells were infected with HSV-1 and treated with prostaglandin A_1 (PGA_1 30 μM) or

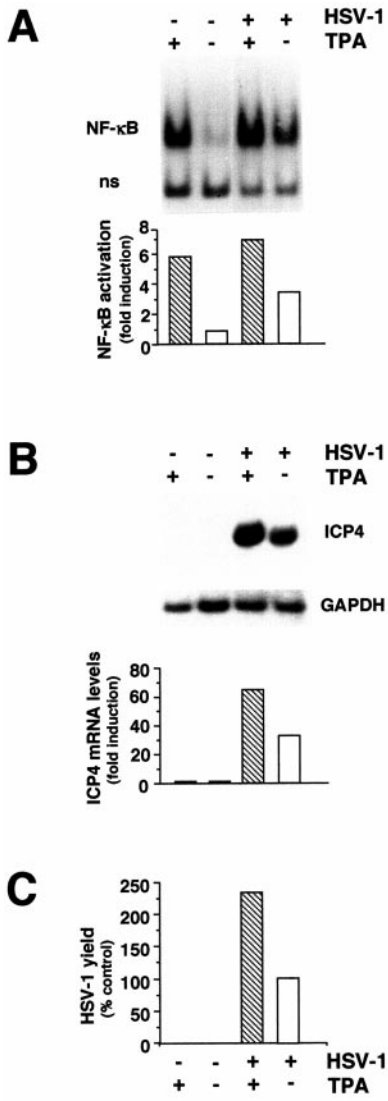

FIG. 3. **Activation of NF-κB stimulates HSV-1 replication.** *A,* mock-infected (−) and HSV-1-infected (+) HEp-2 cells were treated with TPA (25 ng/ml) soon after the 1-h adsorption period. Eight hours p.i., whole-cell extracts were analyzed by EMSA for NF-κB activation (*upper panel*). Section of a fluorogram from the native gel is shown. Positions of NF-κB-DNA (*NF-κB*) and a nonspecific protein-DNA (*ns*) complexes are indicated as in Fig. 1. Levels of NF-κB DNA-binding activity in uninfected or HSV-1-infected HEp-2 cells untreated (−*TPA*) or treated with TPA (+*TPA*) were quantitated by Molecular Dynamics PhosphorImager analysis (*lower panel*). NF-κB levels are expressed as fold induction of NF-κB levels in mock-infected, untreated cells. *B,* in the same experiment, cytoplasmic RNA from duplicate samples were extracted at 8 h p.i. and analyzed for ICP4 mRNA levels by Northern blot analysis (*upper panel*, *ICP4*). Levels of GAPDH mRNA in the same samples are shown as control (*middle panel*, *GAPDH*). ICP4 mRNA levels were quantified by Molecular Dynamics PhosphorImager analysis and expressed as fold induction as compared with the levels in mock-infected cells (*lower panel*). *C,* in the same experiment, virus yields in triplicate samples of untreated or TPA-treated HSV-1-infected cells were determined by $CPE_{50\%}$ assay at 8 h p.i. Data are expressed as percentage of virus yield in untreated cells.

control diluent soon after the 1-h adsorption period. At different time intervals, cell extracts were analyzed for NF-κB activation by EMSA, IκBα degradation by immunoblot analysis, and IKK activity by kinase assay. As shown in Fig. 4, *A* and *B*, treatment with PGA_1 was effective in inhibiting IKK activity and preventing IκBα degradation and NF-κB activation by HSV-1. PGA_1 treatment did not inhibit HSV-1-induced activation of c-Jun amino-terminal kinase (JNK) in the same samples, indicating that PGA_1 targets a selected signaling pathway utilized by the virus. PGA_1 also completely prevented HSV-1-induced NF-κB-dependent transcription in cells transiently transfected with the 2xNF-κB-LUC reporter (Fig. 4*C*).

A single treatment with PGA_1 prevented HSV-1-induced NF-κB activation up to 24 h p.i. (Fig. 5*A*). The effect of PGA_1 on viral mRNA accumulation, protein synthesis, and infectious virus production was then determined. HEp-2 cells were infected with HSV-1 and treated with 30 μM PGA_1 soon after the 1-h adsorption period. Mock-infected cells were treated identically. PGA_1 toxicity was analyzed in uninfected and HSV-1-infected cells by microscopic examination and MTT assay. Levels of ICP4 mRNA were determined at 8 h p.i. by Northern blot analysis. Cellular and viral protein synthesis was determined at 24 h p.i. by SDS-PAGE and autoradiography, after [^{35}S]methionine labeling. At the concentration of 30 μM PGA_1 did not alter cell viability up to 48 h after treatment and did not inhibit cell protein synthesis in uninfected or HSV-1-infected cells (data not shown). As shown in Fig. 5, *C* and *D*, treatment with PGA_1 resulted in a dramatic decrease of viral gene expression and completely prevented HSV-1 protein synthesis. A single PGA_1 treatment caused over 3000-fold reduction in infectious particle production up to 72 h p.i. (Fig. 5*B*).

In a different experiment confluent monolayers of HEp-2 cells were infected with HSV-1 and treated with different concentrations of PGA_1 1 h after infection. Levels of NF-κB-DNA binding activity were determined 8 h p.i. in cellular extracts. In parallel samples virus yield in the supernatant of infected cells was determined 24 h p.i. As shown in Fig. 6*B*, PGA_1 exerts antiviral activity only at concentrations that inhibit NF-κB. PGA_1 antiviral activity is dose-dependent and is correlated with inhibition of NF-κB activation by HSV-1 (IC_{50} for inhibition of NF-κB DNA binding activity = 20 μM; IC_{50} for HSV-1 replication = 18 μM). To investigate whether treatment with PGA_1 started at later stages of infection, when the virus-induced events leading to NF-κB activation have already taken place, is still effective in inhibiting HSV-1 replication, HEp-2 cells were infected with HSV-1 and treated with PGA_1 (30 μM) at different times p.i. Cell extracts were assayed for NF-κB-DNA binding activity 24 h p.i. At the same time virus titers were determined in the supernatant of infected cells. As shown in Fig. 7*B*, treatment with PGA_1 started in the first 3 h after infection completely suppressed HSV-1 replication and HSV-1-induced NF-κB activation, whereas treatment started at 6 h p.i. was less effective; treatment started at 9 h p.i., when NF-κB activation is maximal, or at later times after infection had no effect on HSV-1 replication, suggesting that inhibition of NF-κB via IKK is an important factor in the antiviral activity of this molecule. Data shown in Fig. 7*A* also indicate that treatment with PGA_1 started at 9 h p.i. or later is not effective in preventing NF-κB activation by HSV-1. This finding further supports the hypothesis that early viral protein synthesis is responsible for NF-κB activation. The inability of PGA_1 to inhibit NF-κB activity when given at late stages of infection could be due to the fact that, as shown in Fig. 4, HSV-1-induced IKK activity appears to reach a peak around 7 h p.i. and then decreases at 11 h after infection, suggesting that IKK activity at late stage of viral infection is not essential; treatment started at this time would then not be effective in inhibiting NF-κB activation. The fact that the activated form of NF-κB can still be detected at 24 h p.i. suggests that HSV-1 infection could affect NF-κB down-regulation, possibly by inhibiting cellular protein synthesis and therefore reducing IκBα production. In fact, we were unable to detect newly synthesized IκBα at late stages of infection up to 24 h p.i. (data not shown).

Inhibition of HSV-1-induced NF-κB Activation and Virus Replication by PGA_1 in Human Neuroblastoma Cells—Herpes

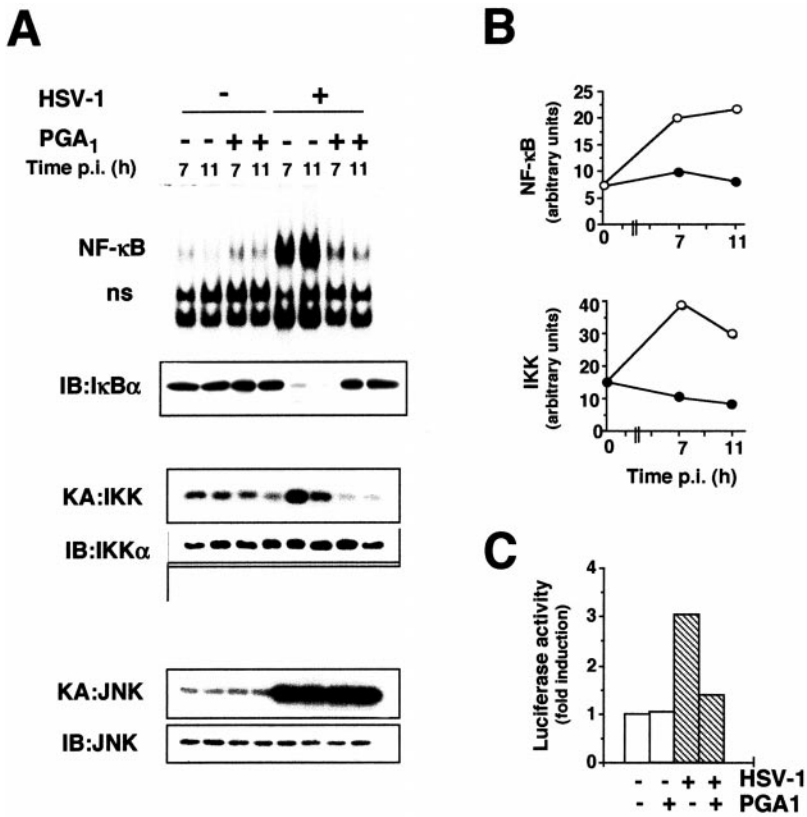

FIG. 4. **PGA_1 inhibits HSV-1-induced NF-κB and IKK activities and NF-κB-dependent transcription.** Mock-infected (−) or HSV-1-infected (+) HEp-2 cells were treated with PGA_1 (30 μM) (+) or control diluent (−) soon after the 1-h adsorption period. *A,* at different time intervals, whole-cell extracts were analyzed for NF-κB activation by EMSA (*top panel*), IκBα degradation by immunoblot analysis (*middle panel*), and for endogenous IKK and JNK activities and recoveries by kinase assay (*KA*) and immunoblotting (*IB*), respectively. *B,* NF-κB and IKK activities in untreated (○) or PGA_1-treated (●) infected cells were quantified by Molecular Dynamics PhosphorImager analysis and expressed as arbitrary units. *C,* HEp-2 cells were transiently transfected with a 2xNF-κB-LUC reporter. After 24 h, cells were infected with HSV-1 (+) or were mock-infected (−) and treated with PGA_1 (30 μM) (+) or control diluent (−) soon after the 1-h adsorption period. Cells were assayed for luciferase activity 12 h p.i. in duplicate samples. The activity achieved in mock-infected untreated cells was given an arbitrary value of 1, and the other values were calculated relative to that.

simplex viruses are known to infect a variety of human cell types. In particular, HSV are able to enter neuronal cells and establish latent infections, which play a major role in the recurrence of HSV-induced lesions. To investigate whether HSV-1 is able to activate NF-κB in neuronal cells, human SK-N-SH neuroblastoma cells were infected with HSV-1 at the multiplicity of infection of 10 pfu/cell, and NF-κB activation was determined at different times p.i. by EMSA. To verify whether PGA_1 was effective in blocking HSV-1-induced NF-κB activation and virus replication in neuronal cells, parallel SK-N-SH cultures infected with HSV-1 were treated with 12 μM PGA_1. Virus titers were determined in the supernatant of infected cells 24 h p.i. Infection with HSV-1 caused a potent and persistent activation of NF-κB in neuroblastoma cells that, similar to the results shown for HEp-2 cells, started at 3 h p.i. and continued for at least 24 h, and was completely prevented by PGA_1 treatment (data not shown). Treatment with PGA_1 caused a significant reduction of virus yield also in neuroblastoma cells (control, $3.15 \pm 0.09 \times 10^6$ $CPE_{50\%}$/ml; PGA_1-treated, $0.12 \pm 0.07 \times 10^6$ $CPE_{50\%}$/ml; $p < 0.01$).

DISCUSSION

Viruses have evolved different strategies to target critical steps in the host cell life, taking over cellular functions to promote their replication. Several viruses take advantage of the preexisting cellular signaling pathways to induce cellular and/or viral gene expression. In the case of herpesviruses, it has been recently shown that HSV-1 activates the JNK/stress-activated protein kinase (SAPK) and the p38 mitogen-activated protein (MAP) kinase, and it has been suggested that activation of these kinases could influence virus replication (22–24).

In the present report we describe how HSV-1 mimics proinflammatory cytokines activating the IκB kinase IKK, triggering IκBα degradation in the early phase of infection, and causing a dramatic and persistent activation of NF-κB in human cells. Activation starts at 3 h p.i., and the level of NF-κB DNA binding activity increases up to 24 h after infection. Levels of IKK activity were also found to be elevated for several hours after infection. Different herpesviruses were reported previously to activate transiently NF-κB independently of gene expression during or immediately after virus adsorption, through a viral gB- and gH-receptor-dependent mechanism involving a TNF receptor-associated factor (TRAF)-mediated signaling pathway (13). We have shown that in human cells HSV-1-induced NF-κB activation is persistent and is dependent on *de novo* synthesized proteins. The fact that the anti-herpetic drug acyclovir, which does not affect the synthesis of viral IE and DNA replication-independent proteins, was found to have no effect on HSV-1-induced NF-κB activation indicates that the synthesis of immediate-early or early viral gene products is necessary for NF-κB induction. These results are in agreement with the previous observation that two IE proteins, ICP4 and ICP27, were required for NF-κB nuclear translocation in C33 cells infected with a different strain of HSV-1 (13, 14). An important role for ICP4 protein not only in NF-κB nuclear translocation, but also in NF-κB activation and triggering NF-κB-dependent transcription, is indicated by a series of experiments, in which HEp-2 cells were transiently co-transfected with the pRB3094 vector (25) containing the HSV-1 *ICP4* gene together with 2xNF-κB-LUC reporter or empty vectors. Results from these studies indicate that ICP4 is able to potently activate NF-κB and NF-κB-dependent transcription in HEp-2 cells in the absence of viral infection (data not shown).

Activation of NF-κB is not specific for HEp-2 cells. We have found that HSV-1 can induce persistent activation of this factor also in HeLa and 293 cell lines, in Jurkat lymphoblastoid cells, as well as in cells of neuronal origin (SK-N-SH) (data not shown). Persistent activation of NF-κB appears then to be a general response of human cells to HSV-1 infection and could play an important role in viral pathogenesis. In fact, induction of NF-κB function is utilized by HSV-1 to enhance its replication, by increasing the efficiency of NF-κB-dependent viral gene transcription. Patel *et al.* (13) had previously shown that

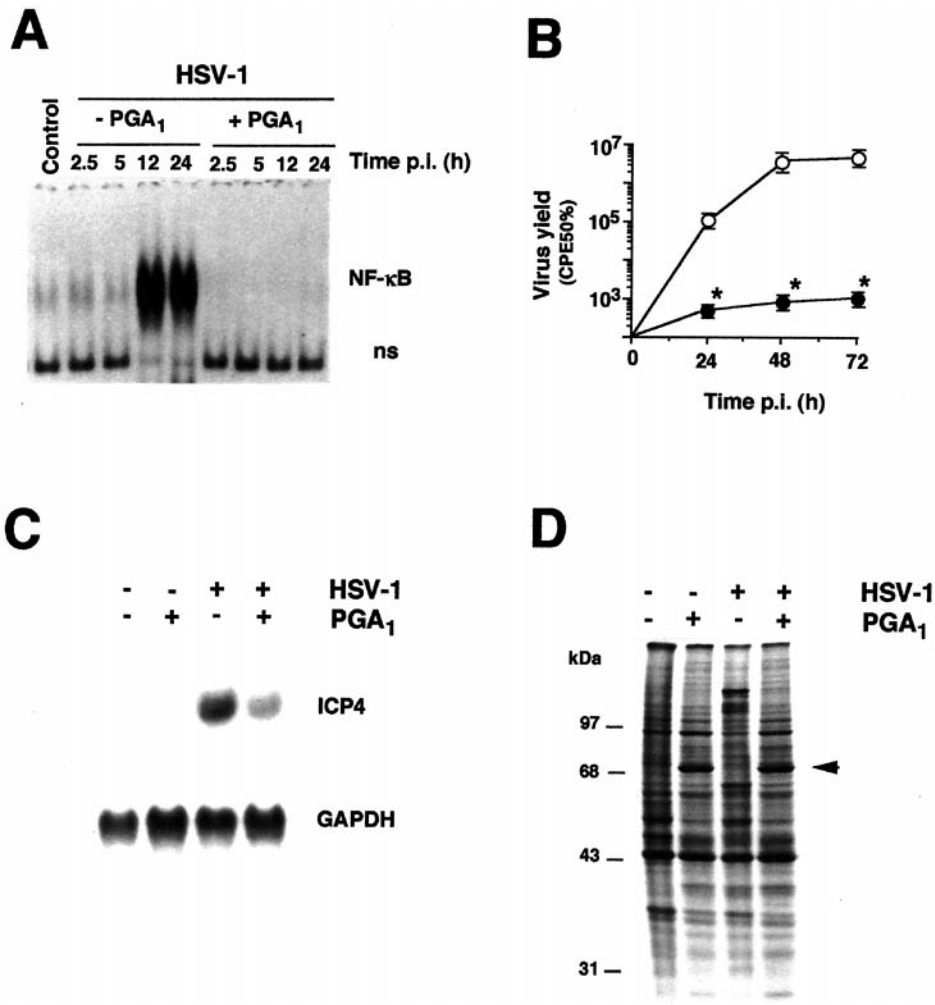

FIG. 5. **Effect of PGA_1 on HSV-1 replication in HEp-2 cells.** Mock-infected (*Control*) or HSV-1 infected (*HSV-1*) HEp-2 cells were treated with PGA_1 (30 μM) (+ *PGA_1*) or control diluent (− *PGA_1*) soon after the 1-h adsorption period. *A,* at different times p.i., whole-cell extracts were analyzed by EMSA for NF-κB activation. *B,* virus yield was determined by $CPE_{50\%}$ assay at different times p.i. in untreated (○) or PGA_1-treated (●) cells. Data represent the mean ± S.D. of duplicate samples of a representative experiment. Each experiment was repeated at least 3 times. *, $p < 0.05$ as compared with untreated control. *C* and *D*, HEp-2 cells mock-infected (−) or infected with HSV-1 (10 pfu/cell) (+) were treated with PGA_1 (30 μM) (+) or control diluent (−) soon after the 1-h adsorption period. Eight hours p.i., cytoplasmic RNA was analyzed for ICP4 mRNA levels by Northern blot analysis (*C, upper panel*). Levels of GAPDH mRNA in the same samples are shown as control (*C, lower panel*). In parallel samples, cells were labeled with [^{35}S]methionine soon after the 1-h adsorption period for the following 24 h. Samples containing equal amounts of radioactivity were processed for SDS-PAGE and autoradiography (*D*). Molecular weights are shown on the *left*. *Arrow* indicates a 70-kDa protein induced by PGA_1 treatment that was identified as the 70-kDa heat shock protein hsp70 by Western blot analysis (data not shown).

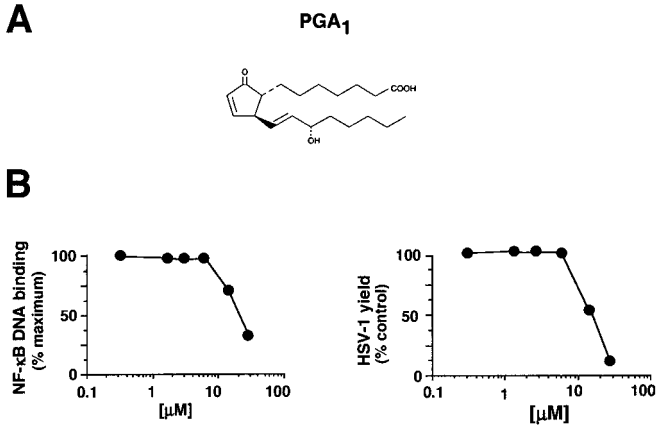

FIG. 6. **Dose-dependent effect of PGA_1 on HSV-1-induced NF-κB activation and infectious particle production in HEp-2 cells.** *A,* structure of PGA_1. *B,* HEp-2 cells infected with HSV-1 were treated with the indicated concentration of PGA_1 or with control diluent soon after the 1-h adsorption period. Eight hours p.i., whole-cell extracts were analyzed by EMSA for NF-κB activation. Levels of NF-κB-DNA binding activities were quantified by Molecular Dynamics PhosphorImager analysis and expressed as percentage of the maximal activity achieved in the absence of the inhibitor (*left panel*). In the same experiment, virus yield in the supernatants of infected cells was determined by $CPE_{50\%}$ assay at 24 h p.i. and expressed as percentage of virus yield in untreated cells (*right panel*). Data represent the average from two separate experiments.

HSV-1-induced NF-κB nuclear translocation is critical for virus replication in C33 cells, as an 80–90% reduction in virus yield was demonstrated following infection of C33 cells expressing a constitutive repressor for IκBα. We have now shown that inhibition of HSV-1-induced NF-κB by transient transfection of HEp-2 cells with the IκBα super-repressor (IκBα-AA) prevents NF-κB-dependent transcription and expression of viral genes and results in a dramatic reduction of virus yield in infected human cells. On the other hand, stimulation of NF-κB with TPA or TNFα results in more than 100% increase in the level of viral mRNA expression and virus yield at early stages of infection (8 h p.i.). Moreover, HSV-1 infection and selected viral proteins have been shown to enhance the transcriptional activity also of heterologous viral gene promoters and, in particular, can activate transcription from the long terminal repeat promoter region of HIV-1 via an NF-κB-dependent mechanism (14, 26). Finally, because of the critical role of NF-κB in promoting inflammation, persistent activation of NF-κB could be a key factor in triggering HSV-1-induced inflammatory processes by stimulating the expression of proinflammatory and chemotactic cytokines (27). IKK and NF-κB could therefore be attractive therapeutic targets for novel anti-herpetic drugs.

Prostaglandins (PG), a class of naturally occurring cyclic 20 carbon fatty acids with potent biological properties, participate in the regulation of a variety of physiological and pathological processes, including the immune response, cytoprotection, inflammation, the febrile response, and virus infection (28, 29). The ability of prostaglandins of the A type to inhibit virus replication and prevent the establishment of persistent infections was first reported in 1980 (30). It is now well established that PG containing an α,β-unsaturated carbonyl group in the cyclopentane ring structure (cyclopentenone PG) possess a potent antiviral activity against a variety of DNA and RNA

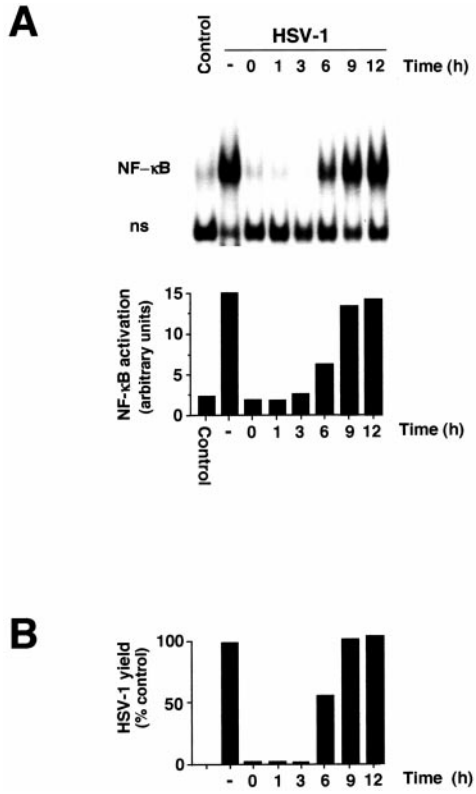

FIG. 7. **PGA_1 inhibits an early event in HSV-1 replication cycle.** HEp-2 cells were infected with HSV-1 (10 pfu/cell) and treated with control diluent (−) or PGA_1 (30 μM) soon after the 1-h adsorption period (time 0) or at 1, 3, 6, 9 and 12 h p.i. At 24 h p.i., whole-cell extracts were analyzed by EMSA for detection of NF-κB activity (*A, upper panel*). Levels of NF-κB-DNA binding activities were quantified by Molecular Dynamics PhosphorImager analysis and expressed as arbitrary units (*A, lower panel*). *Control lanes* represent mock-infected untreated cells. In the same experiment, virus yield in the supernatant of infected cells was determined by $CPE_{50\%}$ assay at 24 h p.i. and expressed as percentage of virus yield in untreated cells (*B*).

viruses in *in vitro* and *in vivo* experimental models (16).

The mechanism of the antiviral activity is distinct from any other known antiviral agent. Cyclopentenone prostaglandins act on the host cell by inducing a cellular defense mechanism, which involves the activation of the heat shock response (31), and can interfere with multiple events during the virus replication cycle (16). In the case of negative strand RNA viruses, cyPG were found to block selectively virus protein synthesis and/or to cause specific alterations in the maturation and intracellular translocation of viral glycoproteins (16). In the case of retroviruses, treatment with PGA_1 or PGJ_2 causes a dramatic block of HIV-1 RNA transcription in human lymphoblastoid cells, whereas it does not affect virus adsorption, penetration, reverse transcriptase activity, nor viral DNA accumulation (21). Inhibition of viral RNA transcription after treatment with the cyclopentenone prostanoids Δ^7-PGA_1 and Δ^{12}-PGJ_2 was also described in human embryonic fibroblasts infected with HSV type 2 (32). However, the mechanism by which cyclopentenone prostaglandins can control viral RNA transcription was not known.

We have now identified the cellular transcription factor NF-κB and the kinase IKK as new targets for the antiviral activity of cyclopentenone prostanoids. In the present report we show that the cyclopentenone prostaglandin A_1 is a potent inhibitor of IKK and NF-κB activation induced by HSV-1 infection. Treatment with PGA_1 started 1 h after infection completely blocked HSV-1-induced IKK activity, preventing IκBα degradation. PGA_1 appears to target a selected signaling pathway utilized by the virus, since it did not inhibit JNK activity which, as reported previously in other cell lines (22–24), was greatly induced in HEp-2 cells following HSV-1 infection. We have shown previously that cyclopentenone prostaglandins block phorbol ester- or TNFα-induced NF-κB activation by inhibition and direct modification of the β subunit of IKK, due to the binding of the prostanoid to cysteine 179 in the activation loop of IKKβ (17). The presence of an α,β-unsaturated carbonyl group in the cyclopentane ring in fact allows cyclopentenone prostaglandins to bind covalently to thiol groups of target cysteine residues of specific proteins via the formation of Michael adducts (16). The fact that PGA_1 does not affect HSV-1-induced JNK activation confirms the previous finding that cyPG do not inhibit JNK1 or p38 activity after exposure to pro-inflammatory cytokines (17). This effect has been ascribed to the lack of a target Michael acceptor cysteine residue in the activation loop of these kinases (17).

NF-κB inhibition by PGA_1 results in the block of HSV-1 gene expression and in a dramatic decrease in the production of HSV-1 infectious particles by HEp-2 cells. A single treatment with PGA_1 at the concentration of 30 μM, which did not inhibit protein or nucleic acid synthesis in HEp-2 cells, caused an over 3000-fold reduction in virus yield up to 72 h after treatment. Similar results were obtained in human neuroblastoma SK-N-SH cells. PGA_1 antiviral activity is dose-dependent and is correlated with inhibition of NF-κB activation by HSV-1. Moreover, treatment with PGA_1 started at later stages of infection, when NF-κB has already been activated by the virus, is ineffective. These results indicate that inhibition of IKK and NF-κB plays an important role in the prostaglandin-induced block of HSV-1 replication. However, since it is known that, like interferon, prostaglandins can act at multiple levels during the virus replication cycle (16), it cannot be excluded that other mechanisms (*i.e.* the activation of the heat shock response) could contribute to the potent anti-herpetic activity of PGA_1.

Different types of prostaglandins are used clinically as cytoprotective drugs for gastroduodenal ulcers, in the treatment of congenital heart disease, erectile dysfunction, and to facilitate labor and are generally effective and well tolerated (33, 34). PGE_1 infusion was also shown to be beneficial in patients with fulminant viral hepatitis (35). The results described suggest that cyclopentenone prostanoids or prostanoid-derived molecules could be good candidates for a new class of antiviral drugs exerting their effect by a novel pathway, inhibiting both virus replication and virus-induced inflammatory stimuli, and suitable for use in combination with conventional anti-herpetic chemotherapy.

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Activation of IκB Kinase by Herpes Simplex Virus Type 1: A NOVEL TARGET FOR ANTI-HERPETIC THERAPY

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