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Targeting the NF-κB pathway through pharmacological inhibition of IKK2 prevents human cytomegalovirus replication and virus-induced inflammatory response in infected endothelial cells

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Abstract

Endothelial cells are important reservoirs for human cytomegalovirus (HCMV) replication, dissemination and persistence. HCMV infection of endothelial cells has been associated with a proinflammatory response characterized by an increased expression of chemokines and adhesion molecules and modulation of angiogenesis. Many of the host proinflammatory genes augmented in HCMV-infected endothelial cells are regulated, at least in part, by the NF-κB pathway. HCMV is a potent activator of NF-κB through the IKK-IκB signaling axis. To explore whether inhibition of HCMV-induced NF-κB activation may interfere with the onset of virus-associated inflammatory response, we measured the effects of the specific IKK2 inhibitor AS602868 on the expression of a panel of proinflammatory genes in HUVEC cells infected with a clinical isolate. Treatment of infected HUVEC with AS602868 was shown to impair HCMV-induced NF-κB activity, IE gene expression, viral replication and to prevent HCMV-induced upregulation of ICAM-1, IL-8, RANTES, IP-10, I-TAC and COX-2 gene expression. Consistent with these results, HCMV-mediated upregulation of another NF-κB-dependent gene, the plasminogen inhibitor type-1, a regulatory factor of endothelial proliferation and angiogenesis, was abrogated by AS602868. These results suggest that inhibition of HCMV-induced IKK-NF-κB activation may be of interest to limit the virus-induced inflammatory response of infected endothelial cells.

Keywords: HCMV; Endothelial cells; Inflammatory response; Gene expression; NF-κB; IKK2

1. Introduction

Human cytomegalovirus (HCMV) is a primary cause of morbidity and mortality in immunocompromised individuals and remains a serious problem in transplant recipients (Mocarski and Courcelle, 2001; Pass, 2001; Landolfo et al., 2003). It has also been implicated in vascular disorders such as transplant vasculopathy, restenosis, and atherosclerosis characterized by endothelial cell activation, inflammatory cell infiltration and smooth muscle cell proliferation (Soderberg-Naucler, 2006). The ability of the virus to infect the vascular endothelium is critical in the pathogenesis of all HCMV diseases (Bissinger et al., 2002; Kahl et al., 2000; Jarvis and Nelson, 2002). In fact, virus dissemination during primary and reactivated infections alike may be promoted by the close interactions between infected endothelial cells and circulating leukocytes that lead to the virus's bidirectional transmission (Gerna et al., 2000). Relevant to these changes is the HCMV's ability to dysregulate endothelial cell gene expression profiles and their activation, differentiation and interactions with other cell types. Hence, dysregulation of inflammatory gene expression and subsequent virus-mediated immunopathogenesis, and persistence of HCMV in large-vessel endothelial cells are all thought to contribute to the pathogenesis of virus-induced vascular damage (Soderberg-Naucler, 2006). Since virus-induced proinflammatory response in endothelial cells has been associated with the activities of viral early functions, current antiviral strategies targeting late events of the HCMV replication cycle have no impact on the changes that may occur independently of virus replication (Craigen and Grundy, 1996).

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Most of the proinflammatory genes upregulated by HCMV are thought to depend, at least in part, on NF-κB activation (Bonizzi and Karin, 2004). The virus itself is a potent NF-κB activator, and the NF-κB pathway is believed to exert a positive role for IE gene expression and viral replication (Yurochko et al., 1995; DeMeritt et al., 2004, 2006). Signals that stimulate NF-κB activation cause the phosphorylation of a class of inhibitor proteins, the IκBs, which sequester the NF-κB dimers in the cytosol of unstimulated cells. Their subsequent dissociation and degradation allow NF-κB proteins to translocate into the nucleus and activate the transcription of host and viral responsive genes (Viatour et al., 2005). The crucial IκBs phosphorylation step is carried out by the IKK (IκB kinase complex) which links the activation of NF-κB proteins to the upstream cellular transduction signal pathways. The "classical" IKK complex consists of two catalytic subunits, IKK1 and IKK2, and the regulatory subunit IKKγ (NF-κB essential modulator, NEMO) (Bonizzi and Karin, 2004). We, and others, have recently demonstrated that HCMV infection of primary fibroblasts stimulates a rapid and sustained increase of IKK activity (Caposio et al., 2004; DeMeritt et al., 2004). Moreover, the activity of the IKK2 subunit was shown to be required for IE gene expression and optimal HCMV AD169 replication in cultures of primary fibroblasts (Caposio et al., 2004).

Since activation of the virus-induced NF-κB pathway is hypothesized to play a role in the development of the HCMV-mediated proinflammatory response of infected endothelial cells, in this study we have investigated the feasibility of blocking IKK2 activity by a specific low molecular weight IKK2 inhibitor (Frelin et al., 2003, 2005) as a mean to prevent NF-κB activation and thus to prevent the development of virus-induced inflammation in an endothelial cell model.

2. Materials and methods

2.1. Cells and viruses

Human umbilical vein endothelial cells (HUVEC) obtained by trypsin treatment of umbilical cord veins were cultured in endothelial growth medium (EGM-2, Cambrex Bio Science, Walkersville, MD). Experiments were performed with cells at passages 2–6. Quiescent HUVEC cells (arrested in G_0/G_1 phase) were obtained by culturing the subconfluent cultures for 96 h in basal EBM-2 medium containing 0.5% FBS and supplements but lacking growth factors (low-serum medium). Low-passage human embryonic lung fibroblasts (HELF cells) were grown as monolayers in minimum essential medium (MEM) (Gibco/BRL) with 10% FBS (Gibco/BRL).

HCMV VR1814 is a derivative of a clinical isolate recovered from a cervical swab of a pregnant woman (Revello et al., 2001). This strain was propagated in HUVEC and titrated by the indirect immunoperoxidase staining procedure on HELF cells using a mAb reactive to the HCMV IE1 and IE2 proteins (clone E13, Argene Biosoft). When infections were performed in the presence of AS602868, the cells were pretreated for 1 h with the compound prior to infection. Following infection, the cells were maintained in the AS602868-containing medium.

2.2. IKK2 inhibitor

AS602868 is an anilinopyrimidine derivative and adenosine triphosphate (ATP) competitor that has been selected for its inhibitory activity in vitro on IKK2ee, the constitutively active form of IKK2. AS602868 acts as a reversible specific IKK2 inhibitor with an IC_{50} of 20 nM toward purified IKK2. It has no effect on the IKK2 relative IKK1 ($IC_{50} = 14\ \mu M$) or on a large panel of recombinant kinases. AS602868 inhibits TNF-α-induced IκB-α phosphorylation and subsequent NF-κB activation in various cell lines. AS602868 also blocked production of TNF-α in LPS-stimulated THP-1 cells as well as ICAM-1 but not ICAM-2 expression in TNF-stimulated HUVEC (Frelin et al., 2003, 2005). Stocks of AS602868 were prepared in 100% DMSO. In all experiments in which AS602868 was used, each dish of cells received an equal volume of DMSO.

2.3. Nuclear extract isolation and electrophoretic mobility shift assay (EMSA)

Quiescent HUVEC cells were infected with HCMV VR1814 at a M.O.I. of 5 PFU/cell in the presence or absence of 10 μM AS602868, or mock-infected. At the indicated times p.i., nuclear extracts were prepared and EMSA was performed with the ^{32}P-labeled double-stranded NF-κB consensus oligo (Promega) as previously described (Caposio et al., 2004). Complexes were analyzed by non-denaturating 4% polyacrylamide gel electrophoresis, dried and detected by autoradiography.

2.4. Immunoblotting

Whole-cell protein extracts were prepared as previously described (Gribaudo et al., 2002). Proteins were separated by SDS/PAGE and then transferred to Immobilon-P membranes (Millipore). Filters were immunostained with goat anti-COX-2 polyclonal antibody (Santa Cruz Biotechnology) (diluted 1:250) or mouse anti-actin mAb (Chemicon) (diluted 1:1000). Immunocomplexes were detected with sheep-anti mouse or rabbit-anti goat Ig Ab conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (Super Signal, Pierce).

2.5. Quantitative viral nucleic acids analysis

Viral DNA levels were measured by quantitative real-time PCR using the previously described probe and primers amplifying a segment of the IE1 gene (Tanaka et al., 2000). HCMV DNA copy numbers were normalized by division with the amount of human 18S rRNA gene (Assay-on-Demand: 18S, assay no. HS99999901_s1, Applied Biosystems) amplified per reaction. Standard curves were constructed using values from the serially diluted genomic DNA mixed with an IE1 encoding plasmid (Gribaudo et al., 2002).

Real-time quantitative RT-PCR analysis (RTQ-PCR) analysis was performed on a Mx 3000 P^{TM} (Stratagene). After HCMV infection and cell treatment, total cellular RNA was isolated using the Eurozol Reagent (Euroclone Ltd., UK) and reverse transcribed. cDNAs or water as controls were amplified in duplicate using the Brilliant SYBR Green QPCR Master Mix

(Stratagene). Primer sequences were: COX-2 (sense, 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′; antisense, 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′); ICAM-1 (sense, 5′-CAA CCG GAA GGT GTA TGA AC-3′; antisense, 5′-CAG CGT AGG GTA AGG TTC-3′); ICAM-2 (sense, 5′-TGA GAC TCT GCA CTA TGA GAC C-3′; antisense, 5′-GCG AGA CAT CAA GTC CAG C-3′); IL-8 (sense, 5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′; antisense, 5′-TCT CAG CCC TCT TCA AAA ACT TCT C-3′); RANTES (sense, 5′-GGG GAT CCA TGA AGG TCT CCG CGG CA-3′; antisense, 5′-CGG AAT TCC TAG CTC ATC TCC AAA GA-3′); IP-10 (sense, 5′-AGC CAA TTT TGT CCA CGT GTT-3′; antisense, 5′-GGC CTT CGA TTC TGG ATT CAG-3′); I-TAC (sense, 5′-GCT ATA GCC TTG GCT GTG ATA TTG TG-3′; antisense, 5′-CTG CCA CTT TCA CTG CTT TTA CC-3′); PAI-1 (sense, 5′-TGC TGG TGA ATG CCC TCT ACT-3′; antisense, 5′-CGG TCA TTC CCA GGT TCT CTA-3′); IE1 (sense, 5′-CAA GTG ACC GAG GAT TGC AA-3′; antisense, 5′-CAC CAT GTC CAC TCG AAC CTT-3′); IE2 (sense, 5′-TGA CCG AGG ATT GCA ACG A-3′; antisense, 5′-CGG CAT GAT TGA CAG CCT G-3′); β-actin (sense, 5′-CAA AAG CCT TCA TAC ATC TC-3′; antisense, 5′-TCA TGT TTG AGA CCT TCA A-3′). For quantitative analysis, semilogarithmic plots were constructed of delta fluorescence versus cycle number, and a threshold was set for the changes in fluorescence at a point in the linear PCR-amplification phase (C_t). The C_t values for each gene were normalized to the C_t values for β-actin with the ΔC_t equation. The level of target RNA, normalized to the endogenous reference and relative to the mock-infected and untreated cells, was calculated by the comparative C_t method with the $2^{-\Delta\Delta Ct}$ equation.

2.6. Antiviral assay

Quiescent HUVEC cells were pretreated with different concentrations of AS602868 for 1 h, and then infected with HCMV VR1814 at a M.O.I. of 1 PFU/cell. Following infection, cultures were maintained in an AS602868-containing medium, and then incubated until control cultures displayed an extensive cytopathology (6 days p.i. for VR1814). Thereafter, the cells and supernatants from the anti-CMV assay were harvested and disrupted by sonication. The extent of VR1814 replication was then assessed by titrating the infectivity of supernatants of cell suspensions on HELF cells by the IE antigen indirect immunoperoxidase staining technique. Plaques were microscopically counted, and the mean plaque count for each drug concentration was expressed as a percent of the mean plaque count of control virus. The number of plaques was plotted as a function of drug concentration; concentrations producing 50 and 90% reduction in plaque formation (IC_{50} and IC_{90}) were determined. Cytotoxicity assays were performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Pauwels et al., 1988).

2.7. Flow cytometry and chemokine ELISA

Quiescent HUVEC cells were pretreated with 10 μM AS602868 for 1 h, and then infected with HCMV VR1814 at a M.O.I. of 5 PFU/cell. After 48 h, the cells were collected, washed twice with PBS containing 1% bovine serum albumin (BSA), and 0.01% sodium azide and then incubated with a primary antibody directed against ICAM-1 (Genzyme) or with an isotype-matched control for 30 min at 4 °C. The cells were then washed twice and incubated with a FITC-labeled goat antimouse IgG1 secondary antibody (Southern Biotechnology) for another 30 min at 4 °C. After washing, the samples were resuspended in PBS-containing 0.01% sodium azide, and at least 5000 events were analyzed on a FACSort (Becton Dickinson). RANTES, IL-8, IP-10 and I-TAC (IFN-inducible T cell alpha chemoattractant) protein levels in cell culture supernatants were determined by ELISA kits (Amersham).

2.8. Plasmids, transient transfection and reporter gene assays

Plasmids containing the indicated regions of human COX-2 were phPES2(−1432/+59), phPES2(−327/+59) and the relative mutant constructs, phPES (KBM), phPES (CRM), and phPES (ILM) (Inoue et al., 1995). HUVEC cells were transfected with 3 μg of the reporter vector of interest added to serum-free EGM-2, mixed with 8 μl of Reagent Plus and 8 μl of lipofectamine (Invitrogen), and incubated for 2 h at 37 °C, after which, the medium was replaced with basal EBM-2 medium containing 0.5% FBS. After 48 h, the cells were infected with HCMV VR1814 (M.O.I. 1) in the presence or absence of 10 μM AS602868, or mock-infected. Luciferase activity was measured at 18 h p.i. with a luminometer. Reporter gene activity was normalized to the amount of plasmid DNA introduced into the recipient cells by real-time PCR with the appropriate luciferase and beta actin primers.

3. Results

3.1. Inhibition of IKK2 activity prevents the HCMV-mediated NF-κB activation in HUVEC cells

As shown in Fig. 1, NF-κB activation in HUVEC cells following HCMV VR1814 infection was detectable as early as 15 min from the addition of the virus and remained sustained until 24 h p.i. Treatment with the IKK2 inhibitor prevented this activation up to 24 h p.i., thus demonstrating that inhibition of upstream IKK2 kinase activity was effective in blocking the NF-κB signal transduction pathway elicited by HCMV infection in endothelial cells. Preliminary experiments demonstrated that 10 μM AS602868 entirely prevented the virus-induced NF-κB activation (IC_{50} of 2.5 μM) in quiescent HUVEC cells (data not shown) without affecting cell viability (Fig. 2D).

3.2. The IKK2 inhibitor abrogates HCMV IE gene expression and the replication of a clinical isolate in HUVEC cells

Next, we measured the IE gene expression in HUVEC cells infected with VR1814 in the presence of increasing concentrations of AS602868. As shown in Fig. 2A, about 70% of infected

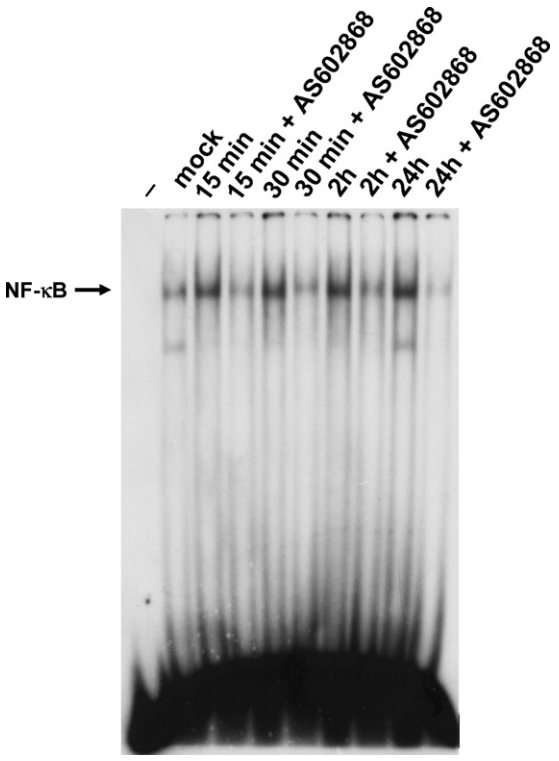

Fig. 1. IKK2 inhibitor AS602868 prevents HCMV-induced NF-κB activation in endothelial cells. Growth-arrested HUVEC cells were infected with HCMV VR1814 (M.O.I. of 5 PFU/cell) or mock-infected. Nuclear extracts were then prepared at the indicated times and assayed for NF-κB activation by EMSA. Where indicated, the cells were pretreated and treated with 10 μM AS602868 1 h prior to and during infection. This experiment was repeated three times, and a representative autoradiogram is shown.

HUVEC cells expressed the two major IE proteins at 96 h p.i. Inhibition of IKK2 activity effected a 100-fold reduction of this expression. Consistent with these results, analysis of IE1 and IE2 mRNAs content demonstrated that the synthesis of both IE mRNAs was significantly decreased by incubation of infected cells with 10 μM AS602868 throughout the entire viral replicative cycle (Fig. 2B). The effects of AS602868 on HCMV DNA synthesis were then evaluated by real-time PCR quantifying at 96 h p.i. the number of viral DNA per μg cellular reference DNA (18S rRNA gene). As shown in Fig. 2C, the number of copies of viral DNA in the control were 6.9×10^5, whereas in the presence of 1 and 10 μM AS602868, the number of viral genomes decreased by two orders of magnitude.

We next examined the effects of IKK2 inhibition on the full VR1814 replication cycle in endothelial cells. Pretreatment with AS602868 1 h before infection produced a significant concentration-dependent inhibition of VR1814 yield at 6 days p.i. (Fig. 2D). The calculated 50 and 90% effective antiviral concentrations (IC_{50} and IC_{90}) were less than 0.01 and 0.1 μM, respectively. A two-log decrease in viral replication was observed even when AS602868 was added as late as 24 h p.i. (data not shown), suggesting that, in addition to IE gene expression, other steps in the HCMV cycle may also be affected by IKK2 inhibition. Moreover, AS602868 did not significantly affect the viability of either quiescent or growing HUVEC cells at concentrations up to 10 μM at 4 days of exposure (Fig. 2D). The extrapolated 50% cytotoxic concentration (CC_{50}) was >30 μM, demonstrating that the antiviral activity of the IKK2 inhibitor was indeed specific and not due to unspecific cytotoxicity.

3.3. Inhibition of IKK2 activity prevents HCMV-induced upregulation of ICAM-1, IL-8, RANTES, IP-10, ITAC-1 and PAI-1 gene expression in infected HUVEC cells

As shown in Fig. 3A, infection of HUVEC cells with VR1814 significantly increased ICAM-1 expression at 48 h p.i., whereas the addition of AS602868 abrogated such an increase. Quantitative real-time RT-PCR demonstrated (Fig. 3B) that the IKK2 inhibitor suppressed the virus-induced increase of the ICAM-1 mRNA content. To address the specificity of this inhibition, the expression of ICAM-2 mRNA (a NF-κB independent gene, whose expression is not modified by HCMV infection of HUVEC cells) (Shahgasempour et al., 1998) was measured in the same RNA samples. As shown in Fig. 3B, AS602868 did not significantly modify ICAM-2 mRNA expression, suggesting that in our experimental setting it specifically inhibits NF-κB signalling pathway without affecting transcription in general.

The extracellular levels of IL-8, RANTES, IP-10 in the supernatants of HUVEC cells infected with VR1814 in the absence or presence of AS602868 were then evaluated. As shown in Fig. 4A, HCMV infection increased IL-8 (3.5-fold), RANTES (6-fold), and IP-10 (17-fold) secretion at 48 h p.i. The secretion of the CXC chemokine IFN-inducible T cell alpha chemoattractant (I-TAC), another chemokine whose expression depends on NF-κB activation (Bitko et al., 2004), but so far not known to be regulated by HCMV infection, was also increased 2.4-fold in the supernatant of virus-infected HUVEC (Fig. 4A). AS602868 pretreatment prevented the virus-induced upregulation of all chemokines examined. As observed for ICAM-1, addition of the inhibitor also blocked the HCMV-dependent increase of IL-8, RANTES, IP-10, and I-TAC mRNA levels (Fig. 4B).

We next investigated whether IKK2 inhibition interferes with HCMV-mediated dysregulation of the proangiogenic plasminogen inhibitor type-1 (PAI-1) (Woodroffe and Kuan, 1998). As shown in Fig. 5, VR1814 infection of HUVEC cells increased PAI-1 mRNA by about three-fold at 24 h p.i. compared with mock-infected cells, while treatment with the IKK2 inhibitor prevented the virus-induced increase in PAI-1 gene expression.

3.4. The IKK2 inhibitor abrogates HCMV-dependent dysregulation of COX-2 gene expression

To determine whether IKK2 inhibition alters HCMV-stimulated COX-2 expression (Zhu et al., 2002), HUVEC cells pretreated with AS602868 were infected with VR1814, and cell extracts were prepared at the indicated times. Immunoblotting analysis (Fig. 6A) showed that the level of COX-2 protein was low in mock-infected cells but began to increase at 24 h and lasted until 48 h p.i. This increase was completely abrogated by treatment with AS602868. As shown in Fig. 6B, consistent with these results COX-2 mRNA content increased in the absence

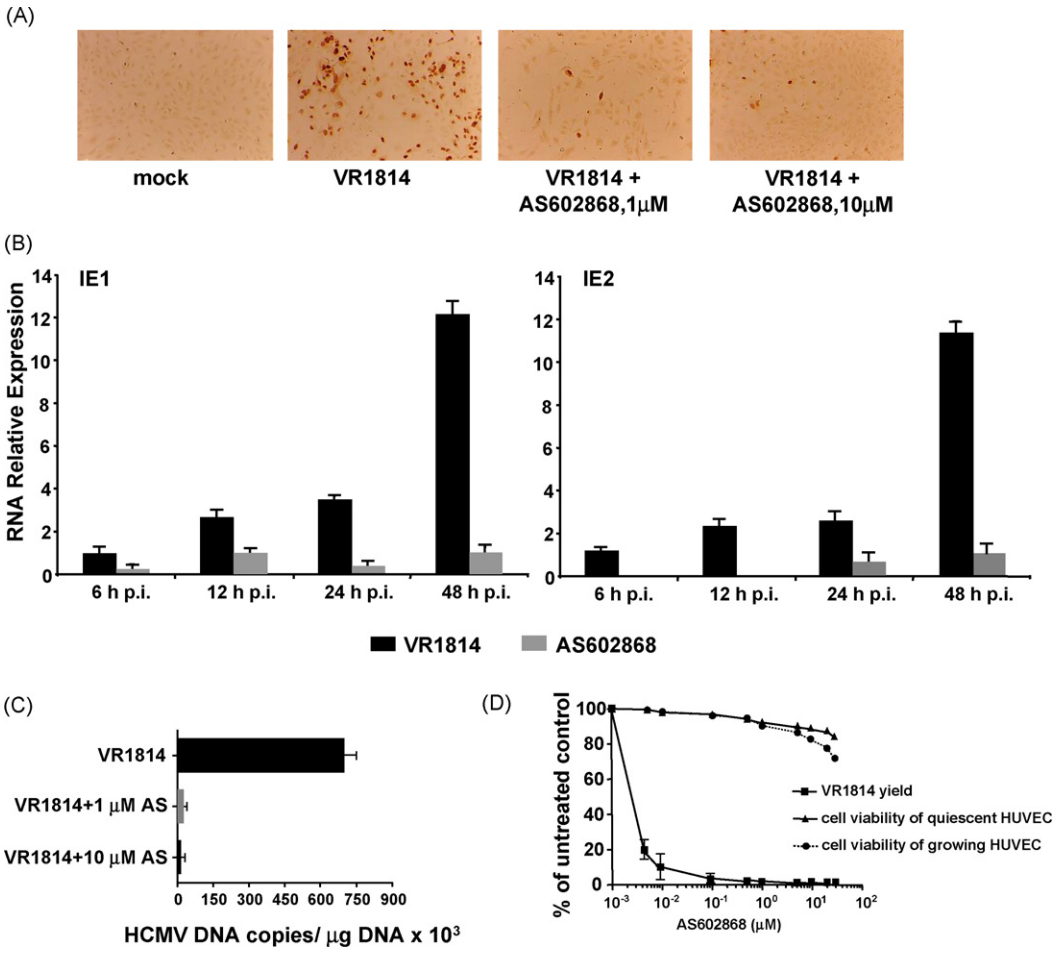

Fig. 2. Influence of AS602868 on HCMV IE gene expression and HCMV replication in endothelial cells. (A) AS602868 inhibits IE protein expression. Quiescent HUVEC cells were infected with HCMV VR1814 (M.O.I. of 1 PFU/cell) or mock-infected. Where indicated, the cells were pretreated and treated with AS602868 1 h prior to and during infection. At 96 h p.i., the cells were fixed, permeabilized and stained with an anti-HCMV IEA mAb. Immunostaining of HCMV IEA in HCMV-infected or AS602868-treated and HCMV-infected cells is shown. The experiment was repeated twice, and representative results are presented. (B) Effects of AS602868 on IE1 and IE2 mRNA expression. HUVEC cells were treated with AS602868 and infected with VR1814 as described before. Total RNA was then isolated at the indicated times after infection and reverse transcribed. RTQ-PCR was then performed with appropriate IE1, IE2 and beta actin primers. For each time point, the IE1 and IE2 RNA levels were normalized according to expression of the actin gene. The data shown are the averages of two experiments ± standard error of the means (error bars). (C) Effect of AS602868 on HCMV VR1814 DNA synthesis. HUVEC cells were treated with AS602868 and infected with VR1814 as described before. Total genomic DNA was then purified at 96 h p.i. and then subjected to real-time PCR. The data shown are the averages of two experiments ± standard error of the means (error bars). (D) AS602868 inhibits HCMV VR1814 replication in endothelial cells. Quiescent HUVEC cells were infected with VR1814 and, where indicated, the cells were pretreated and treated with increasing concentrations of AS602868 1 h prior to and during infection until an extensive viral cytopathic effect was observed in the untreated control. The extent of VR1814 replication was then assessed by titrating the infectivity of supernatants of cell suspensions on HELF cells by the IE antigen indirect immunoperoxidase staining technique. Plaques were microscopically counted, and the mean plaque counts for each drug concentration were expressed as a percent of the mean count of the control. The number of plaques was plotted as a function of drug concentration, and the concentrations producing 50 and 90% reduction in plaque formation (IC_{50} and IC_{90}) were determined. To determine cell viability, quiescent or growing HUVEC cells were exposed to increasing concentrations of AS602868. After 4 days of incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels et al., 1988).

of AS602868 by about four-fold at 24 h versus mock-infected cells, whereas AS602868 blocked the HCMV-stimulated COX-2 mRNA upregulation at 12, 24, and 48 h p.i.

To determine if the increase in COX-2 mRNA was a consequence of stimulation of COX-2 gene promoter activity, we analyzed the effects of HCMV infection on the activity of a transfected luciferase reporter gene driven by different DNA fragments from the 5′-flanking region of the human COX-2 gene (Fig. 7) (Inoue et al., 1995). As shown in Fig. 7, VR1814 infection led to a more than eight-fold increase in luciferase activity in the extracts prepared from cells transfected with the phPES2(−1432/+59) which contained nucleotide −1432 to +59 of the COX-2 gene. The virus-inducible promoter activity of phPES (−327/+59) was about 90% of that of phPES2(−1432/+59), suggesting that sequences between −327 and +59 play a major role in the stimulation of COX-2 promoter activity in response to HCMV infection. These results clearly demonstrate that HCMV regulates COX-2 gene expression in endothelial cells primarily at the transcriptional level.

The presence of a potential NF-κB binding site within the −327/+59 DNA fragment, which acts as a HCMV-inducible promoter (Fig. 7), raised the possibility that this element may

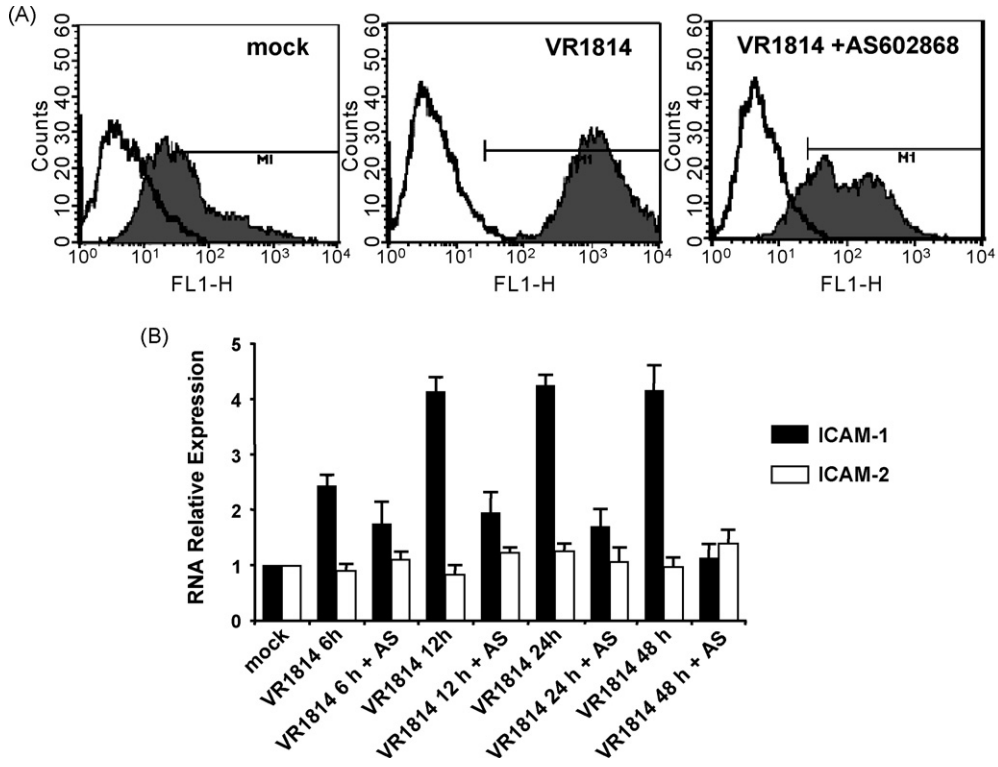

Fig. 3. Effects of AS602868 on HCMV-induced ICAM-1 expression in endothelial cells. (A) AS602868 prevents the increase of ICAM-1 surface expression following HCMV infection. Quiescent HUVEC cells were infected with HCMV VR1814 (M.O.I. of 5 PFU/cell) or mock-infected. Where indicated, the cells were pretreated and treated with 10 μM AS602868 1 h prior to and during infection. The cells were harvested at 48 h p.i. and examined for surface ICAM-1 expression by flow cytometry. The experiment was repeated three times and representative results are shown. (B) AS602868 blocks HCMV-induced ICAM-1 mRNA upregulation. HUVEC cells were treated with AS602868 and infected with VR1814 as described before. Total RNA was then isolated at the indicated times after infection and reverse transcribed. RTQ-PCR was then performed with appropriate ICAM-1, ICAM-2 and beta actin primers. For each time point, the ICAM-1 and ICAM-2 RNA levels were normalized according to expression of the actin gene. The data shown are the averages of two experiments ± standard error of the means (error bars).

contribute to the regulation of promoter activity in response to HCMV infection. To examine this question, reporter constructs phPES2 (KBM) with a mutation at the NF-κB site (−223/−214 bp), phPES2 (ILM) with a mutation at the NF-IL6 site (−132/−124) or phPES2 (CRM) with a mutation at the cAMP response element (CRE) site (−59/−53) were transfected in HUVEC cells. The results (Fig. 7) show that destruction of the CRE or NF-IL6 site had little effect on HCMV-mediated transactivation compared with the parental phPES (−327/+59). In contrast, inactivation of the NF-κB site led to a four-fold reduction in reporter activity, suggesting that the NF-κB element is involved in HCMV-induced COX-2 gene activation. As shown in Fig. 7, AS602868 severely impaired HCMV-induced transactivation of all indicator plasmids, suggesting that the prevention of NF-κB activation blocks the HCMV-induced dysregulation of COX-2 gene.

4. Discussion

Activation of the IKK-NF-κB pathway during CMV infection is thought to exert a positive role in MIEP stimulation and in initiating the viral gene cascade during acute infections or reactivation from latency (DeMeritt et al., 2004, 2006; Hummel and Abecassis, 2002; Simon et al., 2005). Recently, we showed that IKK activity is stimulated following HCMV infection of primary fibroblasts and that the activity of its catalytic subunit 2 is required for optimal HCMV AD169 replication in fibroblasts in culture (Caposio et al., 2004). Here, we have investigated the possibility that inhibiting IKK2 activity can interfere with the replication of an HCMV clinical isolate and the development of virus-induced inflammation in an endothelial cell model. Our results indicate that a small molecule-specific IKK2 inhibitor is able to block HCMV replication in endothelial cells and the onset of virus-induced proinflammatory and proangiogenic responses that significantly contributes to HCMV-associated pathogenesis. These effects are independent of cell type or virus strain as the replication of the AD169 laboratory strain was inhibited in primary human embryonic lung fibroblasts (Caposio et al., 2004). Moreover, they seem to be specific for HCMV, since the replication of HSV-1 and a rhabdovirus such as vesicular stomatitis virus (VSV) was not affected (data not shown).

The IKK2 inhibitor AS602868 was found to block HCMV gene expression at a very early stage, as measured by a significant impairment of IE1 and IE2 expression. Because their expression is essential for progression of the HCMV cycle and because there is growing evidence that their ability to dysregulate host gene expression significantly contributes to the pathogenesis of HCMV diseases, it is not surprising that their inhibition correlates with a block in both HCMV replication and inflammatory host cell response.

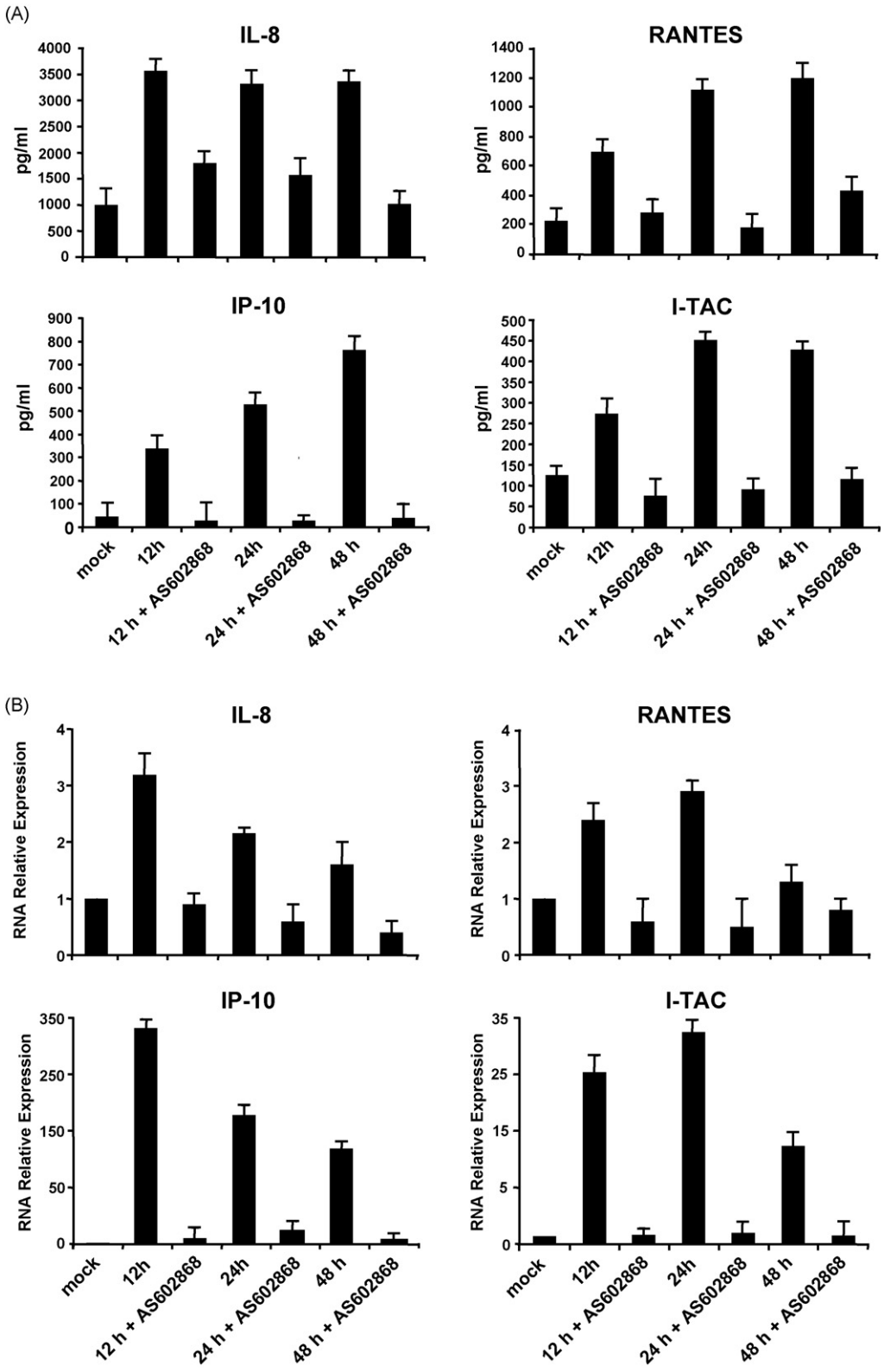

Fig. 4. Influence of AS602868 on upregulation of proinflammatory chemokines in HCMV-infected endothelial cells. (A) AS602868 abrogates HCMV-induced chemokine secretion. Growth-arrested HUVEC cells were infected with HCMV VR1814 (M.O.I. of 5 PFU/cell) or mock-infected. Where indicated, the cells were pretreated and treated with 10 μM AS602868 1 h prior to and during infection. Levels of IL-8, RANTES, IP-10 and I-TAC in the supernatants were then measured at 48 h p.i. The data shown are the averages of three experiments ± standard error of the means (error bars). (B) Effect of AS602868 on HCMV-induced chemokine gene expression. HUVEC cells were treated with AS602868 and infected with VR1814 as described before. Total RNA was then isolated at the indicated times after infection and reverse transcribed. RTQ-PCR was then performed with appropriate IL-8, RANTES, IP-10 and I-TAC and beta actin primers. For each time point, chemokine mRNA levels were normalized according to expression of the actin gene. The data shown are the averages of three experiments ± standard error of the means (error bars).

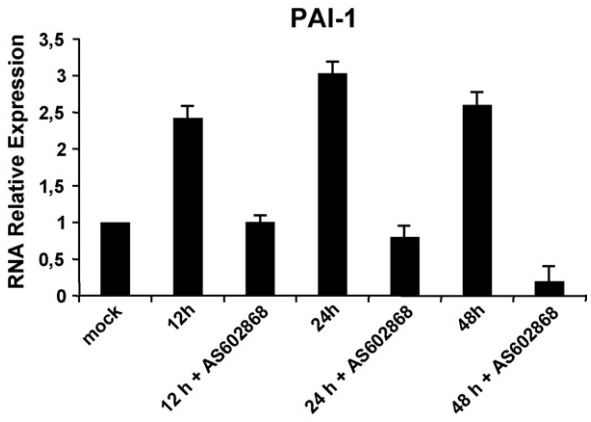

Fig. 5. AS602868 prevents HCMV-mediated upregulation of Plasminogen Inhibitor Type-1 gene expression in endothelial cells. Quiescent HUVEC cells were infected with HCMV VR1814 (M.O.I. of 5 PFU/cell) or mock-infected. Where indicated, the cells were pretreated and treated with 10 μM AS602868 1 h prior to and during infection. Total RNA was isolated at the indicated times after infection and reverse transcribed. RTQ-PCR was then performed with appropriate PAI-1 and beta actin primers. For each time point, PAI-1 mRNA levels were normalized according to expression of the actin gene. The data shown are the averages of three experiments ± standard error of the means (error bars).

The importance of NF-κB in the HCMV replicative cycle has attracted increasing attention (Caposio et al., 2004; DeMeritt et al., 2004, 2006; Prosch et al., 2002, 2003). In these studies, a variety of small molecules acting as inhibitors of different pathway components have been used to test the role NF-κB plays in virus replication. However, concerns in considering this pathway amenable to being targeted as an antiviral strategy are the possibility that its inhibition would be toxic at either the cellular or organism levels and the potential for non-specific effects on virus replication. The proteasome inhibitor MG132, was shown to block HCMV AD169 replication in permissive fibroblasts suggesting that inhibition of proteasome activity could be of interest in anti-CMV chemotherapy (Prosch et al., 2003; DeMeritt et al., 2006). However, the specificity of such inhibitors and their relatively high cytoxic index (the MG132 CC_{50} in primary fibroblasts was about 1 μM) remain a concern, since the proteasome carries out many other important functions and its inhibition could cause severe side-effects. In the current study, the cytotoxicity of the IKK2 inhibitor was not significant in the relevant range of concentrations between 0.01 and 10 μM ($CC_{50} > 30$ μM) in both HUVEC cells and primary fibroblasts. Furthermore, the specificity of AS602868 action was addressed by evaluating the expression of both NF-κB-dependent and NF-κB-independent genes, and the results confirmed that it was able to prevent the virus replication as well as the virus-induced proinflammatory response via NF-κB rather than non-specifically.

Standard therapy for HCMV disease is associated with considerable adverse side-effects since ganciclovir (GCV) may inhibit bone marrow proliferation, and both foscarnet (PFA) and cidofovir (CDV) have been associated with nephrotoxicity (De Clercq, 2003). Moreover, prolonged treatment may lead to the emergence of drug-resistant strains. In addition, these antivirals cannot prevent reactivation of latent HCMV infection or the expression of IE and E proteins. The inhibition of NF-κB activation, viral IE gene expression and the subsequent adhesion molecules and chemokine secretion that we observed in endothelial cells treated with AS602868 may thus

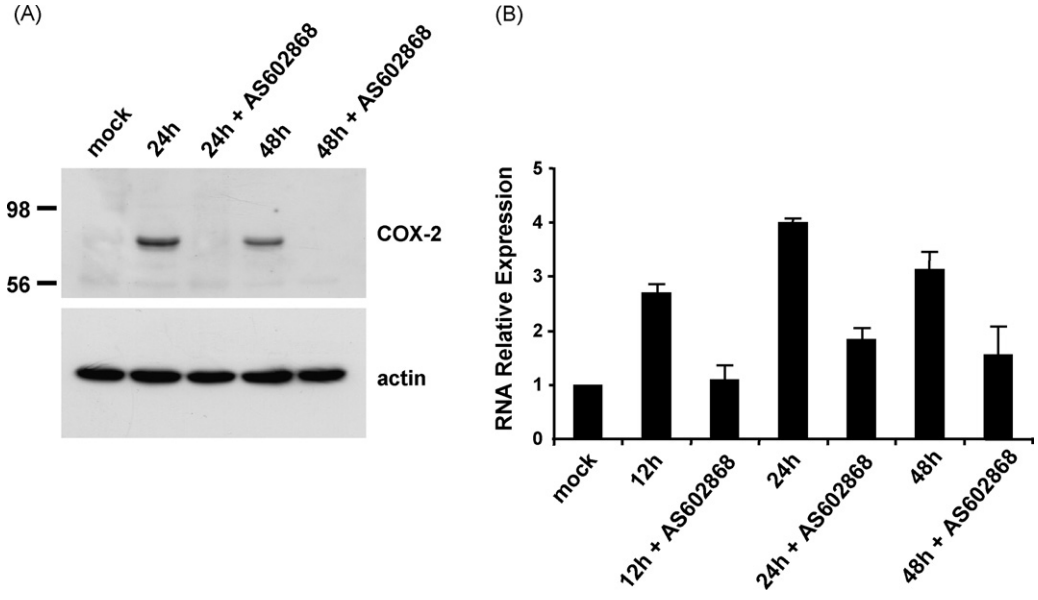

Fig. 6. Upregulation of COX-2 gene expression following HCMV infection is abrogated by IKK2 inhibition. (A) Effect of AS602868 on COX-2 protein expression in HCMV-infected endothelial cells. Quiescent HUVEC cells were infected with HCMV VR1814 (M.O.I. of 5 PFU/cell) or mock-infected. Where indicated, the cells were pretreated and treated with 10 μM AS602868 1 h prior to and during infection. At the indicated times p.i., total cell extracts were prepared and analyzed by immunoblotting with goat rabbit anti-COX-2 polyclonal antibody. Actin immunodetection with mAb was performed as the internal control. (B) AS602868 prevents the HCMV-induced increase of COX-2 mRNA. HUVEC cells were treated with AS602868 and infected with VR1814 as described before. Total RNA was isolated at the indicated times after infection and then reverse transcribed. RTQ-PCR was then performed with appropriate COX-2 and beta actin primers. For each time point, COX-2 mRNA levels were normalized according to expression of the actin gene. The data shown are the averages of two experiments ± standard error of the means (error bars).

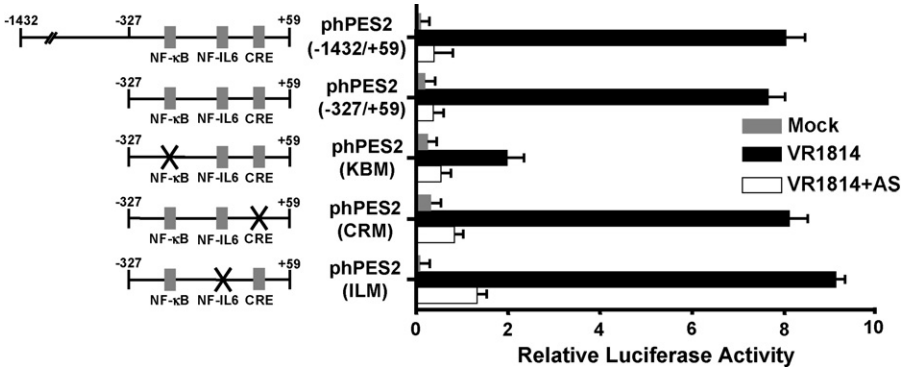

Fig. 7. Involvement of the proximal NF-κB site in the response of the human COX-2 gene promoter to HCMV infection and effect of AS602868 on the virus-mediated transactivation. Each reporter plasmid phPES2 (−1432/+59), phPES2 (−327/+59), phPES2 (ILM), phPES2 (CRM), and phPES2 (KBM) was transfected into HUVEC cells. After 48 h, the cells were infected with HCMV VR1814 (M.O.I. 1) in the presence or absence of 10 μM AS602868, or mock-infected. Luciferase activity was measured at 18 h p.i. The data shown are the averages of two experiments ± standard error of the means (error bars). A schematic representation of the regulatory elements of the human COX-2 gene promoter is shown on the left.

be advantageous to GCV or PFA, since these drugs cannot prevent virus-mediated immunopathogenesis. Relevant to this, in a recent report, AS602868 was found to dose-dependently reduce the expression of RANTES in an ex vivo kidney cultured cell model and to decrease the circulating levels of this chemokine in CD4/HIV1 transgenic mice after 1 or 2 months of treatment (Heckmann et al., 2004).

The importance of IE functions and the inability of currently available antiviral therapy to prevent their expression had led to the suggestion that pharmacological manipulation of their expression and/or functions may provide an alternative strategy to prevent HCMV reactivation, replication and immunopathogenesis (Scholz et al., 2001). What is needed, therefore, is to identify novel anti-cytomegaloviral agents that can block HCMV gene expression in very early stages without causing major adverse side effects. Our results indicate that IKK2 may represent an attractive anti-cytomegalovirus target which may be a good candidate for a new class of antiviral drugs that exert their effect via a novel pathway. IKK2 inhibition reduces both virus replication and virus-induced inflammation, making it suitable for use in combination with conventional anti-cytomegaloviral chemotherapy. Whether these characteristics can be exploited to set up an antiviral regimen *in vivo* remains to be established. To do this, an animal model is required to evaluate whether IKK2 inhibition results in anti-cytomegalovirus activity *in vivo*. Since preliminary *in vitro* experiments have demonstrated that AS602868 inhibits MCMV replication in murine embryo fibroblasts, *in vivo* studies may be helpful to investigate the potential of IKK2 inhibition in the control of HCMV infection.

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