Valproic acid interferes with antiviral treatment in human cytomegalovirus-infected endothelial cells

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Aims The endothelium represents a natural site of human cytomegalovirus (HCMV) infection involved in viral spreading and persistence. Moreover, HCMV infection of endothelial cells has been associated with different pathological conditions of the cardiovascular system. Here, the influence of the antiepileptic drug valproic acid (VPA) was investigated on HCMV replication in human umbilical vein endothelial cells alone or in combination with the antiviral drugs ganciclovir, cidofovir or foscarnet.

Methods and results HCMV replication was observed by immunostaining for viral antigens and by virus yield assay. Protein expression and phosphorylation were examined by western blot. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay. Therapeutic VPA concentrations (≤1 mM) increased HCMV immediate early antigen, late antigen, and viral titres of different endotheliotropic and non-endotheliotropic HCMV strains in a concentration- and time-dependent manner up to 30-fold. Moreover, VPA impaired the antiviral activity of the anti-HCMV drugs ganciclovir, cidofovir, and foscarnet. VPA inhibits histone deacetylases (HDAC) and induces HDAC-independently extracellular signal-regulated kinases 1/2 (ERK 1/2) phosphorylation in endothelial cells. Both effects observed, HCMV stimulation and interference with antiviral drugs, depend on HDAC inhibition but not on ERK 1/2 phosphorylation.

Conclusion These findings suggest to carefully monitor the frequency of HCMV reactivation in cardiovascular patients treated with VPA (or other HDAC inhibitors) in comparison to control individuals.

KEYWORDS

Human cytomegalovirus; Antiviral therapy; Endothelium

1. Introduction

Infection with human cytomegalovirus (HCMV), a ubiquitous herpes virus, results in a life-long latent infection. The virus is a significant pathogen in immunocompromised patients including transplant recipients, neonates, and AIDS patients. HCMV latency is more or less frequently interrupted by reactivations that result in systemic virus spread that often results in serious disease in immunocompromised individuals.1

Endothelial cells are natural sites of HCMV infection. HCMV replicates and causes damages in small blood vessels and capillaries of persons with HCMV disease. Moreover, HCMV infection of endothelial cells may promote haematogenous spread during primary infection or HCMV reactivation.2,3 Moreover, the endothelium was suggested to be a site of HCMV latency and persistence.4,5

The histone deacetylase (HDAC) inhibitor valproic acid (VPA)6 is one of the most frequently prescribed antiepileptic drugs7 and it is becoming the first choice treatment for bipolar disorder worldwide.8 In addition, VPA is clinically used for a number of different further pathologies including schizophrenia and different forms of headache9–11 and clinically investigated as anti-cancer drug.12,13

VPA has been shown to stimulate replication of a number of different viruses including HCMV, Epstein-Barr virus (EBV) human immunodeficiency virus type 1 (HIV-1), human herpesvirus type 6 (HHV-6), human herpesvirus type 8 (HHV-8), measles virus, and poliovirus.14–23 VPA was used in combination with highly active antiretroviral therapy (HAART) for the depletion of latent HIV-1 with contradictory results.19,23 Given the high prevalence of HCMV, ranging from about 60% in developed countries to >90% in other parts of the world,24 and the high number of VPA-treated patients, HCMV-stimulatory effects of VPA would affect a considerable amount of persons.

Here, we investigated the influence of therapeutic VPA concentrations (plasma concentrations have been reported to be ≥1 mM15,25) on HCMV gene expression and replication in human umbilical vein endothelial cells (HUVECs) as well as on efficacy of the anti-HCMV drugs ganciclovir, cidofovir, and foscarnet.

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Valproic acid interferes with antiviral treatment

2. Methods

2.1 Materials

All cell culture supplements were purchased from Biochrom (Berlin, Germany). VPA was obtained from Sigma (Taufenkirchen, Germany). The VPA derivatives 5-2-pentyl-4-pentoic acid and R-2-pentyl-4-pentoic acid were a kind gift of Heinz Nau (Lebensmitteltoxikologie und Chemische Analytik, Tierärztliche Hochschule Hannover, Germany). All VPA derivatives used in the in vitro experiments were dissolved in dimethylsulfoxide (DMSO) to give stock solutions of 1 M. The structurally non-related HDAC inhibitor trichostatin A (TSA) was obtained from Merck Biosciences (Darmstadt, Germany). All cell culture supplements were purchased from Biochrom (Berlin, Germany). VPA was obtained from Sigma (Taufenkirchen, Germany). Foscarnet (Foscavir) was purchased from AstraZeneca GmbH (Wedel, Germany).

2.2 Cells

HUVECs were isolated as described before. Cells were seeded onto Matrigel (BD Biosciences, Heidelberg, Germany; diluted 1:80 in culture medium)-coated culture flasks and grown in Iscove’s modified Dulbecco’s medium (IMDM, Sigma, Taufkirchen, Germany) supplemented with 15% foetal calf serum (FCS), 5% pooled human serum (Blood Bank of The German Red Cross, Frankfurt am Main, Germany), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2.5 ng/mL basic fibroblast growth factor.

The investigation conforms to the principles outlined in the Declaration of Helsinki.

2.3 Viruses

Strain H91 was isolated from the urine of an AIDS patient with HCMV retinitis. The HCMV laboratory strains Towne and AD169 were obtained from American Type Culture Collection (Manassas, VA, USA). The endotheliotropic HCMV strain TB40/E was obtained from Christian Sinzger (Institut für Medizinische Virologie, Tübingen, Germany) and the endotheliotropic clinical isolate VR1814 was obtained from Gabriele Hahn (Max von Pettenkofer-Institut für Virologie, München, Germany). Virus stocks were prepared in human foreskin fibroblasts (HFFs) or HUVEC maintained in MEM with 4% FCS. The titres were determined by plaque titration as described previously.

2.4 Virus infectivity assay

Confluent cultures of HUVEC were incubated with HCMV at different multiplicities of infection (MOI). After incubation for 1 h, which was required for virus adsorption, cells were washed with PBS and incubated in maintenance medium containing 4% FCS. As described in detail previously, cells producing HCMV specific antigens were detected 24 and 96 h post-infection by immunoperoxidase staining using monoclonal antibodies directed against the UL123-coded 72 kDa immediate early antigen 1 (IEA1) (DuPont, Bad Homburg, Germany) and UL53-encoded late antigen gB (LA) (kindly provided by K. Radak, Institut für Virologie, Marburg, Germany), respectively.

2.5 Virus yield assay

The amount of infectious virus was determined by virus yield assay in a single-cycle assay format as described before. In contrast to virus yield assay in fibroblasts where virus titres were determined 3 days post-infection, in HUVEC virus was titrated 5 days after infection. Virus titres were expressed as 50% of tissue culture infectious dose (TCID50).

2.6 Viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as described before in HUVEC. Confuent HUVEC cultures in 96-well microtitre plates were incubated with culture medium containing serial dilutions of VPA derivatives. After 3 days of incubation, MTT (1 mg/mL) was added and after an additional 24 h, cells were lysed in a buffer containing 20% (v/v) SDS and 50% N, N-dimethylformamide adjusted to pH 4.5. Absorbance at 570 nm was determined for each well using a 96-well multispectrometer. After subtracting background absorbance, results are expressed as cell number compared with control cells that were maintained in the presence of solvent. The inhibitory concentration of 50% (IC50) was calculated as the concentration of drug yielding 50% of dye reduction compared with untreated control.

2.7 Cytotoxicity assay

Viable and dead cells were discriminated via staining with 0.4% trypan blue dye. After adding trypan blue to cell suspension, blue stained (dead) and unstained cells were counted in a Bürker chamber and percentage of dead cells was calculated.

2.8 Immunoblotting

Cells were lysed in Triton X-sample buffer and separated by SDS-PAGE, as described previously. Proteins were detected using specific antibodies against β-actin (Sigma, Taufkirchen, Germany), acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY, USA), extracellular signal-regulated kinases 1 and 2 (ERK 1/2) or the phosphorylated forms of ERK 1/2 (each from New England Biolabs, Frankfurt am Main, Germany) and were visualised by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany).

2.9 Statistics

Values presented are the means ± SD of at least three experiments. Comparisons between two groups were performed using Student’s t-test; three and more groups were compared by ANOVA followed by the Student–Newman–Keuls test. P-values lower than 0.05 were considered to be significant.

3. Results

3.1 Influence of valproic acid on HCMV replication in endothelial cells

The effect of VPA in concentrations of up to 1 mM on HUVEC was investigated in growing as well as in confluent cell cultures. Treatment of HUVEC with VPA for 72 h moderately inhibited HUVEC growth but did not result in cytotoxic effects. Treatment with VPA 1 mM resulted in a mitochondrial metabolic activity of 64 ± 9% compared with non-treated control. VPA 0.5 mM did not exert significant effects (95 ± 6% viability compared with control). The MTT-assay does not indicate if this decreased metabolic activity is caused by proliferation inhibition or by cytotoxic effects. Trypan blue staining indicated no statistically significant differences between the number of dead cells in VPA 1 mM-treated cells (2.2 ± 0.6%) and in control cells (3.1 ± 0.9%). In confluent HUVEC, VPA 1 mM treatment for 72 h did not affect cell viability (103 ± 12% compared with untreated control). Trypan blue staining indicated decreased numbers of dead cells after VPA treatment: 1.2 ± 0.6% in VPA-treated (1 mM for 72 h) cells vs. 6.7 ± 1.1% in untreated cells.
Different VPA treatment regimens were tested: incubation prior to infection, addition of VPA during the 1 h adhesion period, or VPA addition after infection. VPA addition during or after virus infection did not influence HCMV Hi91 IEA-expression (data not shown). In contrast, incubation of HUVEC with VPA 1 mM, a concentration that is within the range of therapeutically achievable plasma levels, prior to infection with a MOI of 1 resulted in a strong increase of HCMV Hi91 IEA expression in a time-dependent manner with maximum effects seen after 72 h pre-incubation (Figure 1A). Longer pre-incubation times could not be reliably investigated since the maintenance time of confluent HUVEC in culture is limited.

To investigate, if virus replication cycle continues after IEA expression, HCMV Hi91 LA expression was examined in HUVEC cultures after VPA pre-treatment. VPA treatment increased LA expression in a time-dependent manner, similarly as detected for IEA expression (Figure 1B). Similar results were obtained for HUVEC cultures infected with MOI 10 (data not shown). Further experiments indicated that VPA increases HCMV Hi91 IEA (Figure 1C) and LA (Figure 1D) expression in HUVEC infected with MOI 1 in a concentration-dependent manner after 72 h of pre-incubation.

To examine if VPA increases production of infectious virus, virus titres were determined after pre-incubation for 72 h with VPA 1 mM and infection with HCMV Hi91 (MOI 1). Results revealed more than 10-fold increased viral titres in VPA-treated cells compared with untreated HUVEC (Figure 2).

The influence of VPA on HCMV infection was determined in additional virus strains to investigate if VPA-induced stimulation of HCMV antigen expression may be limited to the clinical isolate Hi91. HUVEC were again pre-treated with VPA 1 mM for 72 h. The non-endotheliotropic laboratory strains AD169 and Towne exerted very low infection efficacy in HUVEC. Therefore, MOI 10 had to be used for these strains. Infection with the endotheliotropic strains TB40/E and VR1814 was performed using MOI 1. VPA treatment resulted in increased IEA antigen expression of non-endotheliotropic as well as of endotheliotropic strains (Figure 3).

### 3.2 Influence of valproic acid derivatives on HCMV replication in endothelial cells

Structurally modified VPA derivatives were used to gain insights in the mechanism of VPA-induced increased HCMV replication in HUVEC. The enantiomers of the 2-pentyl-4-pentynoic acid had been shown before to induce ERK 1/2 phosphorylation in HUVECs. While the S-isomer induced histone hyperacetylation indicating HDAC inhibition, the R-enantiomer did not influence histone acetylation in concentrations of up to 1 mM (Figure 4A).

S-2-Pentyl-4-pentynoic acid decreased HUVEC viability in concentrations >0.5 mM. Therefore, its influence on HCMV Hi91 IEA expression was examined in concentrations of up to 0.5 mM. Pre-incubation of HUVEC for 72 h with S-2-pentyl-4-pentynoic acid increased HCMV Hi91 IEA expression (Figure 4B). Moreover, pre-incubation with S-2-pentyl-4-pentynoic acid also resulted in increased HCMV Hi91 LA expression and virus titres (data not shown). In contrast, R-2-pentyl-4-pentynoic acid did not influence HUVEC viability (data not shown) or HCMV Hi91 IEA expression (Figure 4B) in concentrations of up to 1 mM.

TSA is an HDAC inhibitor that is structurally not related to VPA. As shown before, TSA did not influence on ERK 1/2.
3.3 Influence of valproic acid on HCMV replication inhibition by antiviral drugs

The effects of the three anti-HCMV drugs ganciclovir, cidofovir, and foscarnet were investigated on HCMV antigen expression in VPA-treated and -untreated HCMV Hi91-infected HUVEC. As published before,33 none of the drugs influenced HCMV IEA expression (data not shown). All three drugs reduced HCMV Hi91 LA expression in VPA (72 h pre-incubation)-treated and -untreated cells. However, none of the drugs could fully prevent VPA-induced up-regulation of LA expression. LA expression levels of cells treated with VPA in combination with ganciclovir (10, 20 μM), cidofovir (1, 2 μg/mL) or foscarnet (50, 100 μg/mL) remained above those of the virus control (Figure 5). When added during or after infection, VPA did not interfere with antiviral effects of anti-CMV drugs (data not shown).

To investigate, if VPA-induced ERK 1/2 phosphorylation may contribute to VPA interference with antiviral drugs, the MEK (mitogen-activated protein kinase kinase) inhibitor PD98059 was used. PD98059 10 μM was previously shown to inhibit VPA-induced ERK 1/2 phosphorylation in HUVEC.32 Single treatment with PD98059 (30 min prior to infection) significantly reduced HCMV Hi91 LA expression compared with virus-infected control. However, addition of PD98059 did not significantly reduce VPA-induced increased HCMV Hi91 LA expression and did not influence antiviral effects of ganciclovir. There was also no significant difference between HCMV Hi91-infected cells treated with valproic acid plus ganciclovir and HCMV Hi91-infected cells treated with valproic acid plus ganciclovir plus PD98059 (Figure 6A). The HDAC inhibitor TSA (250 ng/mL) that does not induce ERK 1/2-phosphorylation in endothelial cells (Figure 4C; 35) also prevented antiviral effects of ganciclovir (Figure 6B).

4. Discussion

HCMV-infection of endothelial cells is believed to contribute to different pathological conditions of the cardiovascular system.34 HCMV is suspected to be a co-factor of atherosclerosis,35–37 arterial restenosis,38 acute arterial occlusion,39 post-transplant coronary artery disease,40 vascular endothelial damage after bone marrow transplantation,41 and in allograft rejection or allograft loss after transplantation.41

In this report, VPA is demonstrated to increase HCMV replication in endothelial cells in concentrations of up to 1 mM, which is in the range of therapeutic plasma levels.45,26 VPA 1 mM exerted moderate anti-proliferative but no cytotoxic effects on growing endothelial cells. VPA treatment of confluent endothelial cells did not result in significant differences in cell viability but decreased the number of dead cells. These results are in concert with previous investigations.27,32

Enhanced HCMV antigen expression and replication was only detected after pre-incubation with VPA but not when VPA was added during or after virus infection. Moreover, prolonged pre-incubation times resulted in increased HCMV stimulatory effects. The longest pre-incubation time that could be reliably tested was 72 h. Maintenance of confluent HUVEC for longer periods results in decreased cellular viability. Although HDAC inhibitors are known to exert cell type-specific effects, these findings are in concert with previous investigations showing that VPA increases HCMV replication in HFFs and in human retinal pigment epithelial (RPE) cells in a time- and concentration-dependent manner.20,21

Previous reports had shown that structurally related VPA derivatives increase HCMV replication in strict correlation with their HDAC inhibiting activities.20,21 Here, the S- and R-enantiomer of 2-pentyl-4-pentynoic acid were used to study structure–activity relationships. The S-enantiomer exerts strong HDAC-inhibitory effects in HUVEC, whereas the R-enantiomer does not influence HDAC in concentrations of up to 1 mM in HUVEC. In concordance with previous findings in RPE cells,21 the S-enantiomer increased HCMV antigen expression and replication, whereas the R-enantiomer did not exert significant effects in concentrations of up to 1 mM. Moreover, both enantiomers were able to stimulate ERK 1/2 phosphorylation in HUVEC and ERK 1/2 phosphorylation plays a role in HCMV antigen expression and replication.42,43 However, VPA-induced ERK 1/2 phosphorylation was found to play no dominant role within VPA-induced HCMV-replication in RPE cells.21 Similar results were obtained here. R-2-Pentyl-4-pentynoic acid that was shown previously to induce ERK 1/2-phosphorylation in HUVEC but not to inhibit HDAC15 did not increase HCMV-replication in HUVEC. Moreover, the HDAC inhibitor TSA that does not induce ERK 1/2 phosphorylation stimulated HCMV replication in endothelial cells. These results indicate that HDAC inhibition is the dominant mechanism for VPA-induced enhanced HCMV Hi91 replication, whereas VPA-induced ERK 1/2 phosphorylation does not play a significant role.
VPA was also described to induce phosphorylation of p38 and Akt, two kinases relevant for HCMV replication. However, inhibitors of p38 or Akt did not affect VPA-induced increased HCMV replication (data not shown).

The influence of VPA on anti-HCMV drugs had not been determined before. Three drugs are approved for systemic treatment of HCMV infection: ganciclovir (and its prodrug valganciclovir), cidofovir, and foscarnet. Therapeutic concentrations of VPA (1 mM) that induce ERK 1/2 phosphorylation as well as TSA (250 ng/mL) that does not induce ERK 1/2 phosphorylation interfere with the antiviral effects of all three drugs. These results indicate that inhibition of antiviral activity of the tested drugs depends on HDAC inhibition but not on VPA-induced ERK 1/2 phosphorylation. As shown for stimulation of HCMV replication, pre-incubation with VPA was necessary to affect antiviral action. Consequently, effects on cellular metabolism but not on viral enzymes are most likely to be responsible for impairment of antiviral activity. It is difficult to speculate about or to study the exact mechanism by which VPA detriments the anti-HCMV activities of ganciclovir, cidofovir, and foscarnet. However, it is well established that increased HCMV replication (e.g. through use of higher MOIs) affects the activity of antiviral drugs. Therefore, VPA-induced increased viral replication may be at least in part responsible for the interference of VPA with ganciclovir, cidofovir, and foscarnet. These findings may be important for effective planning of antiviral therapies.

To the knowledge of the authors, no clinical studies have been performed to study the influence of VPA on HCMV replication or on efficacy of anti-HCMV therapy in humans. In immunocompetent persons, stimulatory effects of VPA on HCMV replication would probably not result in clinical symptoms. Nevertheless, a VPA-induced increased frequency of (symptom-free) HCMV reactivations may affect HCMV-associated cardiovascular pathologies. In this context, recent

**Figure 4** Influence of valproic acid (VPA) derivatives and the structurally non-related histone deacetylase (HDAC) inhibitor trichostatin A on human cytomegalovirus expression (HCMV) strain H91 immediate early antigen (IEA) expression in human umbilical vein endothelial cells (HUVECs). (A) Western blots showing levels of acetylated histone H4 (ac. H4) indicating HDAC inhibition in HUVEC treated without (CTL, control) or with VPA (1 mM), S-pentyl-4-pentynoic acid (1 mM), or R-pentyl-4-pentynoic acid (1 mM) for 24 h or expression of ERK 1/2 (ERK) or phosphorylated ERK 1/2 (pERK) in HUVEC after cultivation over night in serum- and growth factor-free medium without (control) or with treatment with VPA (1 mM), S-pentyl-4-pentynoic acid (1 mM), or R-pentyl-4-pentynoic acid (1 mM) for 5 min. (B) HUVEC were treated with VPA, S-2-pentyl-4-pentynoic acid (inhibits HDAC), or R-2-pentyl-4-pentynoic acid (does not inhibit HDAC) for 72 h prior to infection. (C) Western blot showing expression of ERK 1/2 (ERK) or phosphorylated ERK (pERK) in HUVEC after cultivation over night in serum- and growth factor-free medium with different concentrations of the HDAC inhibitor trichostatin A for 5 min. (D) HUVEC were treated with trichostatin A for 24 h prior to infection. *P < 0.05 compared with control; **P < 0.05 for S-2-pentyl-4-pentynoic acid compared with VPA.
findings indicated active HCMV replication in patients with coronary disease. Therefore, the frequency of HCMV reactivations should be monitored and compared between VPA-treated and control collectives in the context of cardiovascular diseases. Moreover, since VPA is a very frequently prescribed drug, there is a fraction of VPA-treated immunocompromised HCMV-positive individuals at risk of HCMV disease. Especially, these patients should be closely monitored for increased frequency and severity of HCMV disease and for the efficacy of antiviral therapy in comparison to a control collective not receiving VPA.

In conclusion, therapeutic VPA concentrations enhance HCMV replication and impair activity of the antiviral drugs ganciclovir, cidofovir, and foscarnet in endothelial cells. The underlying mechanism includes HDAC inhibition whereas VPA-induced ERK 1/2 phosphorylation does not play a dominant role. HCMV infection of endothelial cells is believed to contribute to different pathological conditions of the cardiovascular system. Moreover, endothelia from different tissues may be sites of HCMV persistence. These findings together with data indicating that HDAC inhibition was already shown to induce lytic HCMV replication in monocytes, another proposed site of HCMV persistence, suggest to study the frequency of HCMV reactivation in persons treated with VPA (or other HDAC inhibitors) in comparison to control individuals.

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References


