Sodium valproate, an anticonvulsant drug, stimulates human immunodeficiency virus type 1 replication independently of glutathione levels

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Since modulation of the glutathione (GSH) level has been implicated in the regulation of human immunodeficiency virus (HIV) transcription and expression, we have undertaken an analysis of the effect of sodium valproate (VPA) on HIV-1 replication. VPA, which is an anti-epileptic drug in widespread use in clinical medicine, has been shown to depress the activity of GSH reductase, an enzyme required for maintaining high cellular levels of reduced GSH. The effect of this drug on HIV-1 replication has been studied in primary infected cells, i.e. peripheral blood mononuclear cells (PBMC) and monocyte/macrophages, in the CEM-SS cell line, and in chronically infected stimulated and non-stimulated U1 cells. We have shown that VPA markedly enhanced viral replication in all infected cells tested. Virus production was induced in U1 cells by VPA treatment and the stimulatory effects of tumour necrosis factor-α, interleukin-6 and granulocyte/macrophage colony-stimulating factor were augmented. The LTR-driven gene expression in Jurkat T cells was increased. However, the elevated viral production did not correlate with the effect of VPA on the intracellular GSH level. Thus, VPA stimulated in vitro HIV-1 replication in acutely and chronically infected cells and enhanced LTR-driven gene expression. These effects were observed for concentrations that are reached in the plasma of VPA-treated patients. Therefore, although the clinical significance of these data remains to be demonstrated, these results should be considered in the choice of an anticonvulsant drug in HIV-infected individuals.

Introduction

Valproic acid (n-dipropylacetic acid) is a simple branched-chain fatty acid that has broad-spectrum anticonvulsant activity (Chapman et al., 1982). Its sodium salt, valproate (VPA), is now widely used for the treatment of many forms of epilepsy and various seizure diseases in clinical medicine. Severe adverse effects, mainly related to the liver, have been observed during VPA treatment (Dreifuss et al., 1987). Studies in animals and in humans have shown that after VPA administration there are alterations of glutathione (GSH) metabolism which could in part explain these side effects. In rats, the administration of VPA in association with carbamazepine decreases the level of reduced GSH in the cerebral cortex (Attaguile et al., 1992). VPA also depresses in a dose-dependent manner the activity of rat liver GSH reductase, an enzyme which plays a crucial role in maintaining appropriate cellular levels of GSH (Cotariu et al., 1990). The GSH reductase activity is also reduced in red blood cells of VPA-treated children and in human erythrocytes incubated in vitro with VPA (Cotariu et al., 1992).

Glutathione in its reduced (GSH) and oxidized (GSSG) forms is the major thiol redox system in the cell. GSH acts as a radical scavenger, providing protection against oxidative damage (Meister, 1989, 1991). In human immunodeficiency virus (HIV)-infected individuals, the level of GSH, as well as the levels of other acid-soluble thiols, are decreased in the plasma, in T lymphocytes and in the lung epithelial-lining fluid (Buhl et al., 1989; Eck et al., 1989; Staal et al., 1990, 1992). By
increasing the GSH level, as obtained after exposure of cells to N-acetyl-l-cysteine (NAC) or to GSH esters. HIV replication can be inhibited (Roederer et al., 1990, 1991; Kalebic et al., 1991). GSH precursors also inhibit activation of the NF-κB transcription factor and expression of a reporter gene under the control of the HIV LTR, a promoter containing binding sequences for NF-κB (Mihm et al., 1991, 1995). However, there is no direct evidence that a decrease of the GSH level controls of the HIV LTR, a promoter containing binding sequences for NF-κB transcription factor and induces HIV LTR-mediated replication in the chronically infected monocytic U1 cell line (Legrand-Poels et al., 1990). Moreover, H2O2 also activates the NF-κB transcription factor and induces HIV LTR-mediated transcription (Schreck et al., 1990).

To analyse the effect of a decrease of the GSH level on HIV replication, we have studied virus multiplication in the presence of VPA, an inhibitor of GSH reductase. We report here that VPA markedly enhances viral replication in acutely infected cell lines and human primary cells, and induces virus expression in the chronically infected U1 cell line. However, we could not find any correlation between the increase of virus replication induced by VPA and the GSH level that was valid for all the systems we studied. These observations were made for VPA concentrations that are regularly found in the plasma of treated patients.

**Methods**

- **Cell lines and primary cells.** The Jurkat human lymphoblastoid T cell line, the CEM-SS lymphocytic T cell line, the U937 promonocytic cell line and the HIV-1 chronically infected U1 cell line were cultivated in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS), 2 mM-glutamine, 100 IU/ml penicillin G and 100 µg/ml streptomycin. The U1 cells were stimulated by tumour necrosis factor-α (TNF-α) (100 U/ml), interleukin-6 (IL-6) (50 U/ml) or granulocyte/macrophage colony-stimulating factor (GM-CSF) (30 U/ml), purchased from Genzyme. Rabbit anti-human TNF-α polyclonal antibodies (Genzyme) were used at 2 µg/ml. The U1 and the CEM-SS cells were obtained respectively from Dr Thomas Folks and from Dr Peter Nara through the AIDS Research and Reference Reagents Program, Division of AIDS, NIAID, NIH (Bethesda, USA).

Human peripheral blood mononuclear cells (PBMC) were prepared from whole heparinized blood from healthy HIV-I-negative blood donors, by density-gradient centrifugation on Ficoll (Eurobio). They were isolated by centrifugal elutriation of PBMC and plated in culture dishes. Non-adherent cells were removed 2 h later. The adherent monocyte/macrophages were cultured for 1 week in AIM V medium (Gibco), 2 mM-glutamine and 100 U/ml GM-CSF (Genzyme) until they differentiated into macrophages. The monocyte/macrophages were removed from the dishes using a rubber policeman and plated at a density of 1 x 10^6 cells/ml in 48-well plates (250 µl/well).

Cell viability was measured by trypan blue dye exclusion, by incorporation of [3H]thymidine in infected cells and by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Mossman, 1983).

- **Infection with HIV and VPA treatment.** The CEM-SS cells and PBMC were infected with the HIV-1 LAI strain at 20 and 50 TCID₅₀ respectively, determined by evaluating the reverse transcriptase (RT) activity released from CEM-SS, 5 days post-infection (p.i.). After virus adsorption for 30 min, the cells were washed and cultured at a density of 10⁵ cells/ml for CEM-SS cells and of 4 x 10⁵ cells/ml for PBMC in the presence of VPA. VPA was purchased from Sigma and diluted in the culture medium to obtain 0.125–5 mM final concentration. Viral replication was determined by measuring RT activity in the supernatant at 5 or 7 days p.i. for CEM-SS and PBMC, respectively.

Primary human monocyte/macrophages were infected for 2 h with the HIV-1 monocytotropic BaL strain (Gartner et al., 1986) at 10 TCID₅₀, which was determined by evaluating RT activity released from monocytes/macrophages 15 days p.i. Cells were then extensively washed to remove unadsorbed virus. VPA was added to monocytes/macrophages at final concentrations of 0.03–0.125 mM, after viral adsorption and twice weekly, at every change of the culture medium.

U1 cells (2.5 x 10⁶ cells/ml) were treated with 0.125–5 mM VPA just before stimulation by TNF-α, GM-CSF or IL-6. RT activity was measured after 3 days.

- **RT assay.** At various time points p.i., viral replication was determined either by a quantitative detection of RT activity released in the culture medium or by viral antigen production.

For RT detection, 65 µl of culture medium was incubated at 4°C for 10 min with 10 µl of a lysate buffer (0.5% Triton X-100, 0.75 M-KCl, 50 mM-EDTA) in order to dissociate viral particles. RT assays were then performed at 37°C for 60 min by adding 25 µl of a reaction mixture containing 0.2 M-Tris-HCl (pH 7.8), 20 mM-MgCl₂, 4 mM-EGTA, 3 µCi of [3H]thymidine triphosphate (48 Ci/mmol; Amersham), 5 µg poly(rA)–oligo(dT) (Pharmacia). The reaction was stopped with 20 µl of 120 mM-β-mercaptoethanol dehydrate in 60% TCA. Precipitates were collected on a filter with a micro cell harvester (Skatron) and the radioactive measurement was done with a Betaplate liquid scintillation counter (Pharmacia).

Virus antigen production was determined in culture supernatant and cellular pellets using a commercially available ELISA (ELAVIA; Pasteur Diagnostics) which is polyclonal.

- **Plasmids and transient transfection of Jurkat T cells.** For transfection experiments, the pLC-1 plasmid was constructed by introducing the lacZ gene (HindIII/BamHI fragment) from pCH110 (Pharmacia) into a plasmid derived from pBR322 and containing the HIV-1 LTR sequence (XhoI/HindIII fragment). The pHMG-Tat plasmid, provided by M. Mehtali (Transgène, France), contained the tat gene under the control of the hydroxymethyl glutaryl-coenzyme A promoter (Gautier et al., 1989).

Transfections were performed by the DEAE-dextran method (Sambrook et al., 1989) using 5 µg of each plasmid and 10⁷ Jurkat cells. Twenty-four hours after transfection, cells were plated in 96-well microplates and treated with VPA at concentrations varying from 1.25 to 10 nM. Twenty-four hours later, β-galactosidase activity was measured by a fluorescence assay using 4-methylumbelliferyl-β-D-galactoside (MUG) as a substrate, according to the method previously described (Kuntz-Simon & Obert, 1995).

- **Colorimetric assay for reduced GSH.** Between 2 x 10⁶ and 10 x 10⁶ cells were washed twice with PBS, treated with 0.01 M-HCl to
inactivate γ-glutamyltranspeptidase, lysed by freezing and thawing twice and centrifuged to remove debris. The supernatant was deproteinized with 5% 5-sulphosalicylic acid. Then, the intracellular level of GSH was measured using the GSH-400 kit (Bioxytech), according to manufacturer's instructions. Briefly, a chromogenic compound reacts with all mercaptans present in the sample and ultimately an alkaline β-elimination reaction, specific for the substitution product of GSH, leads to the formation of a chromophoric thione which has a maximum absorbance at 400 nm. The results are expressed in nmol GSH/10^6 cells, except for monocytes where they are expressed in nmol GSH/mg protein. Protein concentration was estimated using the Bradford method (Bradford, 1976).

Results

Effect of VPA on acute HIV-1 infection

The effect of VPA on HIV-1 replication was investigated in the T lymphocytic CEM-SS cell line and in human primary cells, PBMC and monocyte/macrophages. CEM-SS cells were infected with 20 TCID_{50} HIV-1 LAI and treated with 0.125 to 1 mM-VPA. We observed that VPA, at non-toxic concentrations, stimulated virus replication, as shown by the detection of RT activity in the culture supernatant 5 days p.i. (Fig. 1). This effect was measurable at a concentration of 0.125 mM-VPA. At 0.25 mM-VPA, RT production was increased by a factor of 3.75 and at a concentration of 0.5 mM, a 5.5-fold increase of the viral yield was observed. The 50% cytotoxic concentration (CC_{50}) of VPA on mock-infected CEM-SS was 2 mM (data not shown). It must be noted that the enhanced production of extracellular RT correlated with an augmentation of extracellular and intracellular viral antigens (data not shown). This rules out the possibility that the effect of VPA was due only to enhanced virus shedding.

VPA also stimulated virus replication in primary cells. In PBMC infected with 50 TCID_{50} HIV-1 LAI and incubated with various concentrations of VPA ranging from 0.03 to 1 mM-VPA, the drug increased viral replication from 0.125 mM and in a dose-dependent fashion (Fig. 1). The CC_{50} of VPA on mock-infected PBMC was 3 mM (data not shown). At 1 mM-VPA, which was the highest concentration without any cellular toxicity, the RT activity measured in the supernatant 7 days p.i., was about fivefold higher than without the drug (Fig. 1).

With viral antigen detection, the effect of VPA appeared at a concentration as low as 0.125 mM and at 1 mM the viral antigen concentration in the supernatant was 11-fold higher than without the drug (data not shown).

In monocyte/macrophages infected with 10 TCID_{50} of HIV-1 Bal, VPA also stimulated viral replication. VPA was added after virus adsorption and then twice weekly at every change of culture medium. The virus production was measured 14 days p.i. The increased RT production was observed at lower concentrations than for PBMC or CEM-SS (Fig. 1). A maximal stimulation was consistently observed with 0.06 mM-VPA which led to an about ninefold increase of the RT activity measured in culture medium. At higher concentrations the stimulation was lower.

The infection of monocyte/macrophages with HIV-1 induces the synthesis of cytokines, in particular TNF-α and IL-6, which stimulate (by an autocrine pathway) viral replication (Molina et al., 1989; Poli et al., 1990a, b). We therefore tested the effect of VPA on production of these cytokines in non-infected monocyte/macrophages. We observed that VPA was unable to induce TNF-α or IL-6 and did not stimulate their production in monocyte/macrophages treated with bacterial lipopolysaccharides (data not shown).

Effect of VPA on HIV-1 production in U1 cells

We also studied the effects of VPA on chronically HIV-1-infected U1 cells. These cells contain two copies of proviral
DNA (Folks et al., 1988). They constitutively produce low levels of virus but large amounts of HIV-1 can be released when cells are stimulated by cytokines such as TNF-α, IL-6, GM-CSF or by pharmacological agents such as phorbol 12-myristate 13-acetate (PMA) (Folks et al., 1988).

VPA induced HIV-1 multiplication in unstimulated U1 cells (Fig. 2). This effect was significant at a concentration of 0.5 mM-VPA. On this cell line, the CC50 of VPA was 10 mM (data not shown). At 5 mM-VPA, the highest concentration without cellular toxicity, RT activity measured in the supernatant 3 days post-treatment was more than 10-fold higher than without the drug. We investigated the possibility that VPA increased viral production by inducing the synthesis of endogenous TNF-α, as is the case for PMA-stimulated U1 cells (Poll et al., 1990b). For this purpose, U1 cells were incubated with a rabbit antiserum directed against recombinant TNF-α, 30 min before the addition of VPA. This antiserum, which completely and specifically neutralizes the effect of TNF-α, did not modify the VPA-induced viral production (data not shown).

In U1 cells, VPA was also able to enhance, in a dose-dependent manner, viral replication induced by IL-6 and GM-CSF (Fig. 2). This effect was particularly striking in the case of IL-6 induction where a VPA concentration of only 0.5 mM increased the viral yield by about 7-fold and we detected a 20-fold increase at 1 mM (Fig. 2). GM-CSF-mediated stimulations were also increased by VPA, but to a lower extent, as 1 mM-VPA induces a 5-fold increase virus production (Fig. 2). Similar augmentation of virus production was also observed when U1 cells were simulated with TNF-α (data not shown).

**Effect of VPA on the LTR-driven gene expression**

VPA also markedly enhanced the HIV-1 LTR-directed expression of the lacZ gene. In Jurkat T cells transiently transfected with the pLC-1 plasmid in the presence of the pHMG-Tat plasmid and treated 24 h post-transfection with 0.125–10 mM-VPA, the synthesis of β-galactosidase increased as a function of the VPA dose (Fig. 3). At a concentration of 10 mM, the enzyme activity detected 24 h post-treatment was approximately 10-fold higher than in untreated cells. A similar effect was observed in CHO cells stably transfected with both previously described plasmids (data not shown).

**Effect of VPA on the cellular GSH level**

In order to established whether or not VPA, which is considered to be an inhibitor of GSH reductase, was able to modulate the intracellular GSH level, we measured the effect of VPA on the amount of GSH in CEM-SS cells, PBMC and human monocyte/macrophages. We observed that VPA treatment had opposite effects on the intracellular GSH level depending on the cells used. In CEM-SS cells, the GSH level was not modified by VPA (Table 1). In PBMC, VPA increased the GSH level at concentrations which led to a stimulation of viral replication (0.125 to 1 mM). In monocyte/macrophages, a decrease of the GSH level is only observed with high concentrations of VPA.

**Discussion**

We have shown here that VPA was able to stimulate HIV-1 replication. This effect was observed in the continuous CEM-SS cell line as well as in primary cells. PBMC and monocyte/macrophages infected with low amounts (10–50 TCID50) of virus. For CEM-SS and PBMC, the augmentation of virus production was detected at concentration ranging from 0.125–1 mM-VPA. For monocyte/macrophages however, the augmentation was observed at lower concentrations of VPA (0.03 and 0.06 mM), but in this case, addition of VPA to the cultures was more frequent. We ruled out the possibility that VPA stimulates the release of viral particles from the cells without modifying the production of virus constituents, since...
Table 1. Effect of VPA on the intracellular GSH level

The GSH levels were measured 3 days after the addition of VPA. ND, Not done.

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<thead>
<tr>
<th>VPA (mM)</th>
<th>CEM-SS</th>
<th>PBMC</th>
<th>Monocytes</th>
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<tr>
<td></td>
<td>GSH concentration</td>
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<td></td>
<td>nmol/10^6 cells</td>
<td>Variation (%)</td>
<td>nmol/10^6 cells</td>
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<td>4.25</td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td>8.6</td>
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<td>2.4</td>
<td>-11</td>
<td>ND</td>
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<td>9.9</td>
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an increased amount of viral antigens was found both in the culture supernatants and associated with the CEM-SS cells. We checked that VPA had no direct effect on cell proliferation as VPA treatment did not increase [3H]thymidine incorporation in U1 or CEM-SS cell lines (data not shown). VPA also stimulated viral production in the chronically HIV-1-infected U1 cells, which express nearly undetectable levels of virus, and enhanced viral expression in these cells stimulated by IL-6, GM-CSF, and TNF-α. Our results indicate that VPA probably did not enhance viral expression by inducing the production of endogenous TNF-α. VPA acts directly or indirectly (via one of its metabolites) on a step of the viral replication cycle which occurs after the integration of the viral DNA. The expression of the viral genome could be such a target since VPA stimulated the expression of the lacZ gene under the control of the HIV-1 LTR in transiently transfected Jurkat T cells.

VPA has been reported to reduce the GSH reductase activity in rat liver and in human blood cells (Cotariu et al., 1990, 1992). This enzyme reduces GSSG to GSH and plays a crucial role in maintaining high cellular levels of GSH. Therefore, we checked whether a decrease in the amount of the cellular GSH could be correlated with the effects of VPA on viral replication. VPA depressed the GSH level in Jurkat T cells and in U937 cells (from which the U1 cell line was derived) at concentrations which stimulated LTR-driven gene expression or viral replication (Simon et al., 1994). However, the GSH decrease observed in Jurkat T cells and U937 is perhaps not the consequence of the GSH reductase inhibition since Nordhoff et al. (1994) reported that they could not reproduce the previously described effects of VPA on GSH reductase activity.

In CEM-SS cells, the GSH level was not modified by VPA at concentrations which stimulated viral multiplication. In PBMC however, VPA increased the GSH level at concentrations which enhanced viral replication. Previous studies have shown that compounds which lead to an increase in GSH level such as NAC or glutathione esters decrease HIV replication in U1 cells (Roederer et al., 1991), in PBMC (Roederer et al., 1990) and in monocyte/macrophages (Ho & Douglas, 1992). In our study, although an increase of GSH level was observed after treatment of PBMC with VPA, viral multiplication was not decreased; on the contrary it increased. Consequently, whatever the effect on intracellular GSH, VPA increases HIV replication showing that the perturbation of the intracellular redox cycle cannot solely explain the effect of VPA and other mechanisms of action may be involved.

VPA could act through the production, during its metabolism, of free radicals that have been shown to enhance HIV replication (Pace & Leaf, 1995). Indeed, in addition to glucuronidation, this branched-chain fatty acid is predominantly metabolized by β-oxidation and secondarily by ω and ω-1 oxidations (Porchaut & Veitch, 1993; Vamecq et al., 1993; Van den Branden & Roels, 1985). Although the β-oxidation pathway of VPA seems to be different from that of natural unbranched fatty acids, it involves NADPH oxidases. These enzymes lead to production of ROS which could activate the NF-κB transcription factor and viral replication (Legrand-Poels et al., 1990). The fact that VPA stimulated the cytomegalovirus (CMV) immediate early promoter (Kuntz-Simon & Obert, 1995) and the SV40 early promoter (unpublished data), both of which contain NF-κB recognition sequences (Sambucetti et al., 1989; Sassone-Corsi et al., 1984), does not prove (but is compatible with) this hypothesis. Another mechanism of action of VPA which does not involve an oxidative stress and which is independent from NF-κB activation has also to be considered. VPA could act similarly to sodium butyrate, another short-chain fatty acid which, in contrast to VPA, is unbranched. Sodium butyrate has been shown to modulate the expression of several cellular and viral genes (Prasad & Sinha, 1976; Kruh, 1982) and stimulates the replication of herpes viruses (Ash, 1986; Saemundsen et al., 1980; Tanaka et al., 1991) and also of HIV-1 (Bohan et al., 1989; Laughlin et al., 1993, 1995). We have also reported that VPA, like sodium butyrate, increases the replication of CMV in human fibroblasts and transactivated the HCMV IE promoter (Kuntz-Simon & Obert, 1995). Despite a
large number of reports on the effects of sodium butyrate on cellular and viral gene expression, little is known about its mechanism of action, apart from its inhibition of histone deacetylase (Boff et al., 1978). Further work is needed to see whether VPA, like sodium butyrate (Laughlin et al., 1995), stimulates HIV-1 replication by modifying chromatin structure.

VPA is widely prescribed for the management of epilepsy and other seizure diseases. When used in monotherapy, the range of the therapeutic concentrations typically found in the plasma varies from 50 to 100 µg/ml (347–694 µM) (Levy & Shen, 1989; Capek & Esplin, 1990). VPA clearly stimulates in vitro HIV-1 replication in the two major cell types infected in humans: in lymphocytes at the concentrations reached in the plasma and in monocyte/macrophages at lower concentrations. It also promotes viral production in chronically infected U1 cells and enhances the viral yield when these cells are stimulated by cytokines. VPA-treated epileptic patients may be infected by HIV. Additionally, convulsions can occur in AIDS patients, mainly in the case of encephalitis and intracerebral lymphomas. Although the clinical significance of the data presented here has not been established, the fact that VPA enhances in vitro HIV replication should be considered in the choice of an anticonvulsant drug in HIV-infected individuals.

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References


