Antiplatelet Activity of Valproic Acid Contributes to Decreased Soluble CD40 Ligand Production in HIV Type 1-Infected Individuals

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CD40L is a type II membrane glycoprotein of the TNF family that is found on activated T cells, B cells, and platelets. We previously reported that the soluble form of this inflammatory mediator (sCD40L) is elevated in the plasma and cerebrospinal fluid of HIV-1–infected, cognitively impaired individuals. In this study, we demonstrate that the mood-stabilizing drug valproic acid (VPA) reduces sCD40L levels in plasma samples of HIV-1–infected patients (n = 23) and in washed human platelets, which are the main source of circulating sCD40L. VPA also inhibited HIV-1 transactivator of transcription-induced release of sCD40L and platelet factor 4 in C57BL/6 mice. The mechanism by which VPA was able to do so was investigated, and we demonstrate that VPA, a known glycogen synthase kinase 3β (GSK3β) inhibitor, blocks platelet activating factor-induced activation of glycogen synthase kinase 3β in platelets in a manner that alters sCD40L release from platelets. These data reveal that VPA has antiplatelet activity, and they convey important implications for the potential of VPA as an adjunct therapy not only for cognitively impaired patients with HIV-1 infection, but also for numerous inflammatory diseases for which such antiplatelet therapies are currently lacking. The Journal of Immunology, 2011, 186: 584–591.

CD40L (formerly known as CD154) is a type II membrane glycoprotein of the TNF family that is found on activated T cells, B cells, and platelets (1). Classically, CD40L serves as a co-stimulatory molecule expressed on activated CD4+ T cells that binds to its receptor, CD40, on the surface of APCs to induce activation. Binding of CD40L to CD40 on the surface of monocytes, for example, results in enhanced survival and secretion of cytokines, such as TNF-α, IL-1, and IL-6 (1). Cleavage of CD40L produces a truncated form that is soluble, sCD40L, which retains its biological activity and acts as a cytokine (1–3). Activated platelets are thought to be the major source of circulating sCD40L and are estimated to produce nearly 95% of the plasma sCD40L pool (4). Platelets and sCD40L are implicated in several inflammatory diseases, including cardiovascular disease, ischemia/reperfusion injury, and cerebral malaria (4–9). Increased numbers of activated platelets are also found in HIV-1–infected individuals (10), an event that leads to accumulation of sCD40L in the circulation regardless of highly active antiretroviral therapy followed by platelet decline (11, 12). Interestingly, a larger study within the North-East AIDS Dementia cohort indicated that the individuals with declining platelet counts are at greater risk for developing HIV-1–associated neurocognitive disorders (HAND) (13). In this context, sCD40L is present at significantly higher levels in both plasma and cerebrospinal fluid samples of HAND patients (14). Our group previously reported that the mood-stabilizing drug valproic acid (VPA) may have the potential to serve as an adjunct therapy for HAND, demonstrating a trend toward improved cognitive performance when tested in a controlled pilot patient study (15). Along these same lines, we also demonstrated neuroprotective effects of VPA in a mouse model of HIV-1 encephalitis (16). In this study, we now report that VPA reduces sCD40L levels in the plasma of HIV-1–infected individuals. Furthermore, we show that HIV-1 transactivator of transcription (Tat) induces a significant increase in sCD40L levels in C57BL/6 mice, an effect that is abolished in the presence of VPA. Our findings also suggest that the actions of VPA involve inhibition of sCD40L release from purified human platelets.

Previous reports indicate that VPA acts as a nonspecific inhibitor of glycogen synthase kinase 3β (GSK3β) (15, 17), a multifaceted kinase involved in numerous cell processes and known to be present in platelets (18, 19). GSK3β is targeted by platelet activating factor (PAF) (20), which is upregulated during HIV-1 infection (21), and is thought to play a role in cytoskeletal rearrangement and lamellipodia formation in some cell types (22, 23). Based on this, we hypothesized that GSK3β is playing a similar role in platelets, as cytoskeletal rearrangement is an important step in platelet activation and, as such, may be the mechanism through which VPA reduces sCD40L levels. Consistent with this notion, we demonstrate that treatment of human platelets with VPA reverses PAF-induced GSK3β activation. Furthermore, we also demonstrate that cytoskeletal rearrangement is required for sCD40L release, and that treatment of washed human platelets with GSK3β inhibitors...
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attenuates platelet spreading and, therefore, cytoskeletal rearrangement. Collectively, these findings highlight the potential of VPA as a candidate adjunct therapy in HAND, warranting further investigation.

Materials and Methods

Reagents and Abs

HIV-1 Tat1-72 was obtained from Philip Ray (University of Kentucky, Lexington, KY); carbamyl platelet-activating factor (cPAF; a non-hydrolyzable analog of PAF), VPA, LiCl, fibrinogen, BSA, thrombin, cytochalasin E, and Tyrode’s salts solution were all purchased from Sigma-Aldrich (St. Louis, MO); prostacyclin (PGI2) was obtained from Cayman Chemical (Ann Arbor, MI); recombinant GSK3β was purchased from New England Biosciences (Ipswich, MA); and recombinant GST was purchased as described (24). Abs against total GSK3β, phosphoSer9-GSK3β, total protein kinase C (PKC), phospho[Bl Ser465]-protein kinase C (pan), hemagglutinin, and α-tubulin were purchased from BD Transduction Laboratories (San Jose, CA), Cell Signaling Technology (Danvers, MA), and Santa Cruz Biotechnology (Santa Cruz, CA); FITC-conjugated anti-human CD40L, and phallolidin-Alexa Fluor 488 Abs were obtained from Ancell (Bayport, MN) and Invitrogen (Carlsbad, CA), respectively.

Patient material

sCD40L levels were analyzed in the plasma of HIV-1–infected individuals using ELISA. These patients (n = 23) were recruited in a previous study in which blood samples were periodically drawn before and after the treatment (19); plasma samples were cryopreserved (25). The demographics, baseline clinical variables, and inclusion and exclusion criteria of the study subjects have been described (25). The baseline clinical variables of patients include viral load <400 copies/ml and mean CD4+ cell count of 443 ± 223.5 cells/µl. All patients were on a stable antiretroviral regimen containing efavirenz and/or nucleoside reverse transcriptase inhibitors for at least 4 wk before and during the entire period (7 d) of these studies as described (25). All patients gave written consent for all procedures, which were approved by the University of Rochester Research Subjects Review Board.

Isolation of human platelets

Whole blood was obtained from healthy male and female donors, under University of Rochester Institutional Review Board approval and with written informed consent in accordance with the Declaration of Helsinki, by venipuncture into vacutainer tubes containing buffered citrate sodium (BD Biosciences, Franklin Lakes, NJ). Whole blood was then sequentially centrifuged to collect a purified platelet concentrate as described (26). Platelet purity was determined to be >99%.

ELISA

sCD40L was measured in plasma samples derived from HIV-1–infected individuals or supernatants from purified human platelets (9 × 10⁷ cells/sample) using a human CD40L ELISA kit (R&D Systems, Minneapolis, MN) as outlined earlier (14). The concentrations of sCD40L (pg/ml) are presented as a mean ±SEM of indicated replicates for each sample. The values were then compared by a t test, with p < 0.05 indicating statistical significance.

Nine-week-old male C57BL/6 mice (n = 4/group) were given i.p. injections of either water (American Remedies, Shifley, NY) or VPA (200 mg/kg body weight) once a day for 3 d, as previously validated (16). Three hours after injection on the third day, 25 mg/kg HIV-1 Tat was i.v. injected into the tail vein of each of the mice and, following 1 h incubation, blood was collected via cardiac exanguinations. Whole blood was sequentially centrifuged and platelet-poor plasma was collected. Soluble CD40L or platelet factor 4 (PF4) concentrations were measured in platelet-poor plasma samples using a mouse sCD40L ELISA (Bender Med Systems, San Diego, CA) or a PF4 ELISA kit (R&D Systems). Samples were compared using one-way ANOVA followed by Bonferroni’s test for multiple comparisons, which indicated statistical significance as p < 0.05.

All animal experiments were carried out in accordance with the Animal Welfare Act and the National Institutes of Health guidelines. The animal protocol was approved by the University Committee on Animal Resources (University of Rochester Medical Center). The facilities and programs of the Vivarium and Division of Laboratory Animal Medicine of the School of Medicine and Dentistry are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and are in compliance with state law, federal statute, and National Institutes of Health policy.

Platelet counts

Nine-week-old male C57BL/6 mice (n = 5/group) were given fresh water, or that which was supplemented with 3.2 mg/ml VPA, for 18 d. Following treatment, mice were bled from the retro-orbital sinus and counts were performed using a CBC-Diff veterinary analyzer (Heska, Fort Collins, CO).

Tail-bleeding assays

Eight-week-old male C57BL/6 mice (n = 5/group) were treated with VPA in their drinking water as described above. Following treatment, mice were anesthetized and placed on a raised platform with tails protruding over the edge. Tails were positioned 5 mm above filter paper and a 2-mm cut was made in the tip of the tail. The bleeding time was recorded from the time bleeding started until it stopped completely. Values were compared using an unpaired t test that indicated statistical significance as p < 0.001.

In vitro kinase assay

Purified human platelets (9 × 10⁷ platelets/sample) were exposed to 20 nM cPAF alone or together with 0.6 mM VPA for 1 h at 37°C. Lysates were prepared and immunoprecipitation was performed using Abs specific to GSK3β. Immune complexes were incubated with substrate, nRFATc1, and 10 mM [γ-32P]ATP. Substrate nRFATc1 was generated by subcloning PCR amplification product encompassing amino acid region of 147–291 of human NFATc1 into the pGEX-4T-3 expression plasmid (Amersham Biosciences, Arlington Heights, IL), followed by expression of GST fusion protein in Escherichia coli and subsequent purification using previously described methods (20). GSK3β activity was measured by the incorporation of [32P] at the GSK3β-sensitive site of the nRFATc1 via densitometric analysis of autoradiograms (ImageJ software; National Institutes of Health, Bethesda, MD). Data represent mean ±SEM derived from two separate experiments performed in triplicate.

Platelet spreading

Purified human platelets (1 × 10⁷ platelets/sample) treated with either VPA or LiCl for 1 h at 37°C were incubated for 45 min on glass coverslips coated with fibrinogen (100 µg/ml) and blocked with 0.5 µg/ml BSA. Platelets were subsequently fixed with 4% paraformaldehyde. Alternatively, platelets were pretreated with VPA for 20 min, incubated on coated coverslips for 45 min, and then posttreated with vehicle, recombinant GST (as a control), or recombinant GSK3β proteins for 1 h prior to fixation or lysis, as previously described (27). Briefly, recombinant proteins were added to pretreated, spread platelets in Tyrode’s buffer containing dimethyl sulfoxide (≤0.1%) as a vehicle for spontaneous uptake, and following 1 h incubation, coverslips were washed to remove recombinant proteins and the cells were lysed. Immunoblotting was subsequently used to verify delivery of recombinant proteins. Spread and fixed platelets were stained using phalloidin-Alexa Fluor 488 (1/200 diluted in PBS with 0.01% triton) for 45 min at room temperature. Cells were mounted and visualized using an Olympus BX51 light microscope (Olympus, Melville, NY). Platelets were counted and analyzed based on their level of spreading, and the percentages of each type of platelet were calculated for each treatment group; briefly, not spread indicates round platelets lacking filopodia, partially spread indicates the presence of filopodia but not lamellipodia, and fully spread indicates the presence of lamellipodia. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons with p < 0.05 as statistically significant (***p < 0.001, **p < 0.01, *p < 0.05).

Scanning electron microscopy

Platelets were treated and spread as outlined above. The coverslips (in a 12-well plate) seeded with platelets were fixed in a 0.1 M sodium cacodylate-buffered, 2.5% glutaraldehyde fixative overnight at 4°C, postfixed in buffered 1.0% osmium tetroxide, rinsed in buffer, dehydrated in a graded series of ethanol to 100%, transitioned into hexamethyldisilazane to three changes of 100% hexamethyldisilazane, and finally allowed to dry overnight in a fume hood. The coverslips were mounted onto aluminum stubs and sputter coated with gold/platinum for 60 s. Digital images were taken with a Gatan imaging system on a Zeiss Supra field emission scanning electron microscopy.
Results

VPA treatment in HIV-infected individuals is associated with a decrease in circulating sCD40L levels

VPA was previously found to be efficacious in both a pilot patient study as well as in an HIV encephalitis mouse model in the context of HAND (15, 16, 25). Based on these results, and on our previous findings that HIV-1–infected individuals with cognitive impairment had increased levels of sCD40L in their plasma and cerebrospinal fluid (14), we tested whether VPA treatment would have an effect on the level of circulating sCD40L in HIV-positive individuals. To do so, plasma samples were collected from 23 HIV-1–infected individuals who were receiving conventional antiretroviral regimens both before (at baseline) and after 7-d VPA administration (250 mg twice a day orally). sCD40L levels were then measured by ELISA. As shown in Fig. 1, patients receiving VPA treatment had significantly lower (~50%) plasma levels of sCD40L at the end of the 7-d treatment course.

VPA blocks HIV-1 Tat-mediated release of sCD40L in mice

To understand the mechanism through which VPA exerts its effect on sCD40L concentrations in HIV-1–infected patients, we examined the ability of VPA to inhibit HIV-1 Tat-mediated release of sCD40L in mice. Nine-week-old C57BL/6 mice (n = 4/group) were given i.p. injections of either saline or VPA for 3 d and, on the third day, 3 h postinjection, Tat or saline was administered i.v. and plasma was obtained. As shown in Fig. 2A, higher levels of sCD40L were detected in Tat-exposed mice, an effect that was abolished in the presence of VPA. Because sCD40L in plasma is thought to arise mainly from activated platelets (4), we speculated that VPA inhibits Tat-mediated platelet activation, thereby reducing the levels of circulating sCD40L. To test this notion, we used the same plasma samples to measure levels of PF4, as PF4 represents a prominent chemokine released from platelets upon activation. Our results showed that VPA administration also blocks the Tat-mediated increase in PF4 levels (Fig. 2B), suggesting an important role for platelets in this process.

Previous reports have indicated that VPA may lead to the development of thrombocytopenia in patients undergoing VPA treatment (28, 29). To verify that the decrease in sCD40L and PF4 that we see in the presence of VPA was a result of the ability of VPA to dampen platelet activation and not due to loss of platelets, and to avoid undesired platelet activation and subsequent platelet loss, which may result from various injection procedures, C57BL/6 mice were given drinking water supplemented with VPA for up to 18 d, during which time platelet counts were performed at regular intervals. Weight and volume of water consumed remained consistent for each group throughout the course of treatment (data not shown). Following oral administration of VPA, we saw no change in platelet numbers, indicating that VPA was able to dampen platelet activation without affecting platelet numbers in this model (Fig. 2C).

Following the 18-d oral administration of VPA, tail bleed experiments were also performed as a method to determine platelet functionality, since several reports have indicated that VPA may contribute to coagulation abnormalities (30–32). Although we did see a significant increase in clotting time, mice receiving VPA were able to stop the bleeding, indicating that platelets were still functional (Fig. 2D).

VPA inhibits sCD40L release from purified human platelets following exposure to PAF

We next tested whether VPA was able to inhibit sCD40L release directly from platelets. Human platelets isolated from healthy donors were treated with potent platelet activators thrombin and cPAF to induce sCD40L release, either alone or in the presence of VPA. In this case, the platelets were exposed to cPAF, since the biological action of Tat partly involves PAF receptor activation and the degree of neurologic dysfunction observed in HIV-1–infected patients correlates with increased levels of PAF in circulation (21, 33). As shown in Fig. 3, both activators, thrombin and cPAF, were able to induce the release of sCD40L; however, this effect was significantly attenuated following treatment with VPA. Note that the most effective dose of VPA used in this assay was equal to or lower than concentrations measured in the plasma of individuals receiving the standard dose of this drug (15, 25).

Mechanism of platelet deactivation by VPA

As previously mentioned, PAF, which is upregulated during HIV-1 infection, also activates GSK3β in mammalian cells. Furthermore, GSK3β is thought to play a role in cytoskeletal rearrangement and lamellipodia formation in some cell types (22, 23), a process that is important during platelet activation and cytokine release. In an effort to determine the mechanism by which VPA reduces the release of sCD40L from platelets, we first sought to determine whether VPA inhibited PAF-induced activation of GSK3β in platelets. Purified human platelets isolated from healthy donors were treated with cPAF either alone or in conjunction with VPA, and GSK3β-specific in vitro kinase assays were performed using a recombinant peptide substrate containing amino acid residues 147–291 of NFATc1 molecules (20). In this study, we report that cPAF stimulates GSK3β activity in platelets in a manner that is blunted by addition of VPA (Fig. 4A). Because the activity of GSK3β is negatively regulated by inducible phosphorylation of serine 9, we performed additional confirmatory immunoblot assays in which the same platelet lysates were used along with phospho(Ser9)-specific GSK3β Abs. Our results suggest that the pretreatment with VPA indeed blocks cPAF–mediated activation of GSK3β in platelets (data not shown). To test whether the effects of VPA and cPAF were specific to GSK3β, the immunoblots were reprobed with phospho (BH Ser660)-specific PKC (pan) Abs (phosphorylation of PKC at either Thr500, Thr641, or Ser660 residues indicates activation of this kinase). As shown in Fig. 4B, we found no change in the phosphorylation status of PKC in either treatment groups, suggesting that the actions of VPA/cPAF are limited to a certain subset of kinases.

Cytoskeletal rearrangement is important for sCD40L release from platelets and is attenuated following VPA treatment

Spreading platelets undergo a morphological change resulting from cytoskeletal reorganization. As platelets adhere to an extracellular matrix, they extend filopodia followed by the formation of lamellipodia, which gives the platelets a flat, “ruffled” appearance (34, 35). A recent study in platelets isolated from healthy donors showed that the pretreatment with cPAF either alone or in conjunction with VPA, and GSK3β-specific in vitro kinase assays were performed using a recombinant peptide substrate containing amino acid residues 147–291 of NFATc1 molecules (20). In this study, we report that cPAF stimulates GSK3β activity in platelets in a manner that is blunted by addition of VPA (Fig. 4A). Because the activity of GSK3β is negatively regulated by inducible phosphorylation of serine 9, we performed additional confirmatory immunoblot assays in which the same platelet lysates were used along with phospho(Ser9)-specific GSK3β Abs. Our results suggest that the pretreatment with VPA indeed blocks cPAF–mediated activation of GSK3β in platelets (data not shown). To test whether the effects of VPA and cPAF were specific to GSK3β, the immunoblots were reprobed with phospho (BH Ser660)-specific PKC (pan) Abs (phosphorylation of PKC at either Thr500, Thr641, or Ser660 residues indicates activation of this kinase). As shown in Fig. 4B, we found no change in the phosphorylation status of PKC in either treatment groups, suggesting that the actions of VPA/cPAF are limited to a certain subset of kinases.

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This spread state for the platelet allows a greater number of interactions at the site of injury and with other platelets (7, 34, 35), while rearrangement allows movement and secretion of intracellular components needed upon activation (35). Based on this and the results described in Fig. 4, we next examined whether VPA treatment altered platelet spreading by modifying cytoskeletal rearrangement via GSK3β inhibition and whether this action of VPA led to the inhibition of sCD40L release from platelets.

Initially, we verified whether cytoskeletal rearrangement was required for the release of sCD40L from platelets. To do so, platelets were exposed to cytochalasin E, an agent that binds to the growing end of actin filaments to prevent further polymerization (36), either alone or together with thrombin or cPAF, and levels of sCD40L were measured in the supernatant via ELISA. As shown in Fig. 2, HIV-1 Tat induces platelet activation in vivo, which is abolished in the presence of VPA. A. The plasma concentration of sCD40L in C57BL/6 mice was measured via ELISA. Upon i.v. injection with HIV-1 Tat, there is a significant increase in the level of sCD40L found in the plasma; however, this is inhibited, to levels that mimic those of mice receiving saline, when VPA is administered once a day for 3 d prior to injection of Tat. B. Platelet activation was verified in the samples described in A by ELISA specific for PF4 (n = 4/treatment group, labeled as i.p. pretreatment/i.v. treatment). The concentrations of sCD40L (pg/ml) and PF4 (ng/ml) are presented as mean ± SEM of three replicates for each sample. Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s test for multiple comparisons. *p < 0.05 compared with NT. C. VPA does not alter platelet counts in mice receiving drinking water supplemented with 3.2 mg/ml VPA for up to 18 d. D, Oral treatment of VPA does significantly slow thrombus formation, as measured by tail bleeds; however, bleeding does eventually stop indicating functional platelets. Values were compared by an unpaired t test. ***p < 0.001. NT, no treatment.

FIGURE 2. HIV-1 Tat induces platelet activation in vivo, which is abolished in the presence of VPA. A. The plasma concentration of sCD40L in C57BL/6 mice was measured via ELISA. Upon i.v. injection with HIV-1 Tat, there is a significant increase in the level of sCD40L found in the plasma; however, this is inhibited, to levels that mimic those of mice receiving saline, when VPA is administered once a day for 3 d prior to injection of Tat. B. Platelet activation was verified in the samples described in A by ELISA specific for PF4 (n = 4/treatment group, labeled as i.p. pretreatment/i.v. treatment). The concentrations of sCD40L (pg/ml) and PF4 (ng/ml) are presented as mean ± SEM of three replicates for each sample. Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s test for multiple comparisons. *p < 0.05 compared with NT. C. VPA does not alter platelet counts in mice receiving drinking water supplemented with 3.2 mg/ml VPA for up to 18 d. D, Oral treatment of VPA does significantly slow thrombus formation, as measured by tail bleeds; however, bleeding does eventually stop indicating functional platelets. Values were compared by an unpaired t test. ***p < 0.001. NT, no treatment.

FIGURE 3. VPA inhibits platelet release of sCD40L. Release of sCD40L from purified human platelets is inhibited by VPA treatment, indicating that VPA affects platelet-derived plasma sCD40L levels. Purified platelets were treated as indicated, and supernatants were collected for use in an ELISA specific for human sCD40L. Activators alone induced a significant amount of sCD40L release, which was inhibited following VPA treatment. sCD40L concentration (pg/ml) is presented as mean ± SEM of three replicates for each sample from one donor representative of multiple donors. *p < 0.05; **p < 0.01. NT, no treatment.

FIGURE 4. VPA reverses PAF-induced activation of GSK3β, but does not alter PKC activity. A, GSK3β activity in human platelets was measured by conducting in vitro kinase assays using GSK3β-specific Abs and a peptide substrate containing a GSK3β-sensitive site derived from NFATc1 molecules. Incorporation of [32P] was determined by SDS-PAGE and densitometric analysis of autoradiograms (insert). As shown, addition of cPAF was able to activate GSK3β in platelets, which is reversed following VPA treatment. Data represent two separate experiments performed in triplicate. **p < 0.01. B, Platelet lysates prepared as in A were also used to determine the phosphorylation status of PKC via immunoblot analyses in which phosho-specific Abs against PKC were used. NT, no treatment.
FIGURE 5. Cytoskeletal rearrangement is necessary for sCD40L release from platelets and is attenuated following treatment with VPA. A. Supernatants from platelets pretreated with cytochalasin E were collected and ELISA analysis was performed. Cytoskeletal disruption with cytochalasin E reduced sCD40L release induced by the potent platelet activator thrombin.

B. Platelet spreading on fibrinogen-coated coverslips is attenuated following treatment with VPA or another well-characterized GSK3β inhibitor, LiCl. Differential interference contrast (DIC; top panels) and SEM (bottom panels) show increasing numbers of platelets that are partially spread with visible filopodia (arrowheads) but not lamellipodia (asterisks). Staining with phalloidin-Alexa Fluor 488 (Phal.) reveals changes in patterns of F-actin fibers (middle panels; original magnification ×100).

C. Quantification of whole images represented in A. ***p < 0.001; ###p < 0.001 compared with the corresponding level of spreading in NT cells.

D. Spread platelets pretreated with VPA and posttreated with Veh., recombinant GST, or GSK3β were quantified based on their level of spreading as in C. Uptake of GSK3β was verified by performing immunoblot analysis in which platelet lysates and GSK3-specific Abs were used. Our results reveal that the addition of exogenous GSK3β was able to reverse the effects of VPA on cytoskeletal rearrangement and spreading of platelets. **p < 0.01; *p < 0.05 as compared with the corresponding condition in the vehicle-treated cells. DIC, differential interference contrast; NT, no treatment; Phal., phalloidin; SEM, scanning electron microscopy; Veh., vehicle.
5A, release of sCD40L from activated platelets was profoundly blocked by cytochalasin E pretreatment, suggesting that the re-structuring of the cytoskeletal network was needed for the release of sCD40L (similar results were obtained following thrombin or cPAF treatments, and for this reason cPAF data are not shown in Fig. 5A).

Finally, we performed platelet spreading assays to test whether VPA exerts an effect on platelet cytoskeletal rearrangement. Platelets were left untreated or exposed to VPA or LiCl, another well-characterized inhibitor of GSK3β, and spread on fibrinogen-coated coverslips to mimic a site of injury. Subsequently, the cells were either exposed to fluorescein-labeled phalloidin (to stain F-actin polymers) or fixed and subjected to scanning electron microscopy. As determined by phalloidin staining (Fig. 5B, middle panel) and scanning electron microscopy (lower panel), platelets that were left untreated spread as expected and appeared large, flat, and ruffled with smooth edges (left panels, lamellipodia marked with asterisks). In contrast, both VPA- and LiCl-treated platelets appeared as if they were unable to spread completely, with visible filopodia, but lacking lamellipodia (right panels, filopodia are denoted as with arrowheads). Quantification of the levels of spreading is shown in Fig. 5C, which indicates that VPA-treated platelets have altered platelet cytoskeletal rearrangement.

To further verify that GSK3β inhibition is the mechanism by which VPA alters platelet spreading and cytoskeletal rearrangement, spread platelets pretreated with VPA, or left untreated, were posttreated with recombinant GSK3β, recombinant GST as a control, or vehicle. Following posttreatment with GSK3β, a significantly larger percentage of platelets were fully spread compared with VPA-treated platelets that received only vehicle as posttreatment. Thus, addition of exogenous GSK3β reversed the effect of VPA on platelet shape change. Addition of GST did not alter platelet spreading and mimicked results seen with vehicle posttreatment. Quantification of this process is shown in Fig. 5D, along with immunoblot analysis to verify uptake of GSK3β by platelets.

**Discussion**

The use of highly active antiretroviral therapy has made a significant impact on the lives of HIV-1–infected individuals. However, poor penetration of the CNS by these therapies has led to the need for adjunct treatments to address the growing number of individuals affected by neurologic consequences of HIV infection. We previously reported the presence of increased circulating levels of sCD40L in HIV-1–infected individuals with cognitive impairment as compared with infected individuals without cognitive impairment (14). Furthermore, in the same report, we demonstrated that CD40L potentiates the ability of HIV-1 Tat to activate microglia and monocytes (14). Therefore, attenuation of sCD40L levels may prove beneficial in the control of this aspect of the disease by helping to ameliorate the harmful effects of the virus within the CNS. In this study, we demonstrate that VPA is able to reduce plasma levels of sCD40L in both HIV-infected individuals and in an in vivo mouse model. Considering the current widespread clinical use of VPA for conditions such as bipolar disorder and epilepsy, these results have important implications for the potential of VPA or similar drugs as adjunctive therapies for treatment of HAND.

HAND is widely thought to be an inflammatory disease, and thus it is fitting that elevated levels of CD40L have been implicated in numerous other inflammatory diseases, including cardiovascular disease (4, 37) and ischemia/reperfusion injury (8). In these instances the interaction of platelet-derived CD40L with endothelial cells is thought to induce inflammation. CD40, the receptor for CD40L, is constitutively expressed on endothelial cells and, upon ligation by CD40L, these cells become more conducive to monocytes that are being recruited in response to the inflammatory signals (7, 38). We speculate that the elevated levels of sCD40L observed in HIV-1–infected, cognitively impaired individuals are contributing to this inflammatory disorder in a similar fashion. Therapeutic targeting of CD40L is an attractive approach for the treatment of inflammatory disorders (39); however, CD40L is an important costimulatory molecule expressed on T cells, and interfering with it could alter immune competence. Current strategies for targeting CD40L include cyclosporine A, an inhibitor of calcineurin that results in decreased CD40L expression in T cells, and anti-CD40L mAbs, both of which have the potential to confer immunosuppression and thus would not be well suited as adjunct therapies in HAND. The ability of VPA to decrease abnormally high plasma levels of sCD40L, without directly interfering with CD40 signaling, would therefore be advantageous in that desirable humoral immune responses would not be negatively affected. This further highlights the potential of VPA as a candidate adjunct therapy for HAND.

Some reports indicate that patients receiving VPA treatment may experience VPA-induced thrombocytopenia (28, 40); however, any thrombocytopenia observed appears to depend on variables such as gender, age, dosage, or low baseline platelet counts (28, 29, 40). Other published reports indicate that the dosage of VPA required to significantly increase risk of developing thrombocytopenia are >40 mg/kg body weight per day (41), considered a high dose. In our animal studies, mice received concentrations well above 40 mg/kg body weight per day, but they had no evidence of thrombocytopenia. This discrepancy could be explained by the fact that mice possess higher platelet counts than do humans, resulting in higher baseline platelet levels (42). The apparent risk of thrombocytopenia seems to vary depending on risk factors, and while this should be considered clinically, the widespread clinical use of this drug, demonstrating safety and tolerability, still makes it an attractive adjunct therapeutic candidate.

To determine the mechanism by which VPA reduces sCD40L release from platelets, we focused on GSK3β, which is involved in numerous signaling pathways and is known to be inhibited by VPA. GSK3β has been implicated in HAND previously, as it was shown to be activated by PAF in neurons (20). Consistent with this notion, in this study we show that activation of GSK3β is also induced by cPAF in platelets. Several reports have indicated that potent platelet activators such as thrombin, ADP, and collagen lead to inhibition, rather than activation, of GSK3β (18, 19, 43). This phosphorylation-dependent inhibition of GSK3β involves activation of the upstream kinases PI3K and protein kinase B/Akt (19, 43). Consistent with these findings, we observed an increase in levels of phosphorylated GSK3β (indicative of inhibition) in response to thrombin treatment in platelets (data not shown); however, treatment with cPAF results in a significant decrease in phospho-GSK3β, suggesting that PAF activates a different signaling pathway to induce activation, rather than inhibition, of this kinase. Interestingly, we also found a significant increase in sCD40L release in response to thrombin, which would seemingly contradict our hypothesis that active GSK3β is playing a role in sCD40L release. However, this paradox may be explained by the fact that there is a great deal of complexity in the regulatory pathways of this kinase. For example, reduced phosphorylation of GSK3β at Ser9 is usually associated with a 30–50% increase in kinase activity, which is apparently sufficient to induce biological effects, such as neuronal apoptosis (44). Numerous signaling mechanisms target only a specific pool of the GSK3β present in the cells because of the subcellular distribution of both GSK3β and each regulatory
molecule. Although GSK3β is traditionally considered a cytosolic protein, it is also present in other cellular compartments, such as nuclei, mitochondria, and membrane lipid rafts (44, 45). An activity status of GSK3β is different in each compartment, such that the kinase moiety present in the nuclei, mitochondria, and lipid rafts is highly active (dephosphorylated at Ser37); in contrast, cytosolic GSK3β is largely inactive. Thus, complete inhibition or activation of GSK3β in response to regulatory signaling events is highly unlikely. Indeed, we do not see complete inhibition of GSK3β in response to thrombin (data not shown). As previously mentioned, HIV-1 infection is associated with an increase in PAF (21), suggesting that platelets could be activated during infection in a manner that would allow aberrant GSK3β activation and therefore facilitate excess sCD40L release.

The data presented in this study suggest that VPA is able to inhibit the release of sCD40L from platelets due to attenuated cytoskeletal rearrangement via GSK3β inhibition. These data are consistent with previous work that indicates that cytoskeletal rearrangement is indeed necessary for sCD40L release (46, 47). Although it is still unclear whether GSK3β is acting directly on CD40L to inhibit its trafficking to the cellular membrane (which occurs prior to its release), or whether its inhibition blocks the formation or movement of other CD40L-containing vesicles to the surface prior to its cleavage, it is clear that platelet shape change is a necessary component of this process and inhibiting this can lead to altered CD40L solubilization.

Our present study is also consistent with the previous findings by Barry et al. (19) who demonstrated an inhibition of platelet activity in vitro by short-term exposure of platelets to several GSK3β inhibitors (including lithium). Additionally, Hayashi and Sudo (48) showed that treatment of platelets with various agents that elevate cAMP levels inhibit GSK3β, thereby blocking platelet activity. Our study, as well as those of Barry et al. (19) and Hayashi and Sudo (48), contrasts somewhat with the findings reported by Li et al. (18), who found that GSK3β works as a negative regulator of platelet function and thrombosis. In their report, they demonstrated that GSK3ββ/− platelets exhibit agonist-dependent aggregation, ATP secretion, and fibrinogen binding, compared with GSK3β+/+ platelets, suggesting that GSK3β suppresses platelet function in vitro (18). There are, however, important differences between our experiments and those conducted by Li and coworkers. These include the fact that we have examined the effect of pharmacologic inhibition of GSK3β on platelet-derived sCD40L levels, whereas Li et al. determined the effect of genetic deletion of this molecule on thrombotic events (18). Additionally, we have focused on the effect of VPA on platelet activation (and GSK3β) by effector molecules associated with HIV-1 infection (mainly Tat and PAF). In contrast, Li et al. studied platelet function in the context of nonpathogenic regulators of GSK3β. Thus, we hypothesize that the pathologic upregulation of GSK3β activity may lead to quite different effects on inflammatory mediators released by platelets.

To the best of our knowledge, our group was the first to report the potential of VPA as an adjunct therapy for HIV-associated cognitive impairment (15), demonstrating not only a trend toward improved cognitive performance but also improvements in measures of brain metabolism when tested in a controlled pilot patient study (15). Along these same lines, neuroprotective effects of VPA in a murine model of HIV encephalitis were also previously reported (16). The results presented in this study demonstrate that VPA is able to reduce plasma levels of sCD40L in HIV-infected individuals and indicate that this action is linked to the ability of VPA to inhibit GSK3β in platelets. The use of VPA in this context may confer therapeutic benefits not only for HAND, but also other inflammatory diseases, such as stroke, that are linked to platelet activation.

Acknowledgments

We thank Dr. Neil Blumberg for helpful comments on this work. We are also grateful to the University of Rochester Microscope Research Core, specifically Karen L. de Mesy Bentley and Gayle Schneider. We thank the University of Rochester Division of Laboratory Animal Medicine, specifically Robin Westcott. Additionally, we thank Randall M. Rossi for use of the Heska Veterinary Analyzer, as well as Dr. Jamie Bernard, Ann Casey, and Stephen Pollock for valuable assistance.

Disclosures

The authors have no financial conflicts of interest.

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