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Inhibition of Histone Deacetylase Activity by Valproic Acid Blocks Adipogenesis*

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Adipogenesis is dependent on the sequential activation of transcription factors including the CCAAT/enhancer-binding proteins (C/EBP), peroxisome proliferator-activated receptor γ (PPARγ), and steroid regulatory element-binding protein (SREBP). We show that the mood stabilizing drug valproic acid (VPA; 0.5–2 mM) inhibits mouse 3T3 L1 and human preadipocyte differentiation, likely through its histone deacetylase (HDAC) inhibitory properties. The HDAC inhibitor trichostatin A (TSA) also inhibited adipogenesis, whereas the VPA analog valpromide, which does not possess HDAC inhibitory effects, did not prevent adipogenesis. Acute or chronic VPA treatment inhibited differentiation yet did not affect mitotic clonal expansion. VPA (1 mM) inhibited PPARγ induced differentiation but does not activate a PPARγ reporter gene, suggesting that it is not a PPARγ ligand. VPA or TSA treatment reduced mRNA and protein levels of PPARγ and SREBP1a. TSA reduced C/EBPα mRNA and protein levels, whereas VPA only produced a decrease in C/EBPα protein expression. Overall our results highlight a role for HDAC activity in adipogenesis that can be blocked by treatment with VPA.

Valproic acid (VPA)[1] has been used as an anticonvulsant agent for the treatment of epilepsy, as well as a mood stabilizer for the treatment of bipolar disorder, for several decades. Overall VPA is well tolerated, with common side effects including tremor, sedation, alopecia, and weight gain (1). The mechanism of action for these efficacious and deleterious effects remains to be elucidated and is most likely multifactorial. The specific effects of VPA on neurotransmitter systems, most notably γ-aminobutyric acid signaling, as well as other cell signaling pathways, explain some of the mechanisms by which VPA exerts its effects in humans, rodents, and cell culture systems (2). For example, VPA has histone deacetylase (HDAC) inhibitory effects that are the likely cause of VPA teratogenicity (3, 4). In addition, inositol depletion contributes to VPA inhibition of sensory neuronal growth cone formation *in vitro* (5).

Up to 70% of adult patients receiving VPA treatment gain weight (5–14 kg) (6). This is cause for concern because of the increasing number of off label uses for VPA, such as migraine headache and neuropathic pain, and the increased health risk and decreased compliance rate associated with weight gain (6–8). Clinically evaluating patient age, sex, familial predisposition to weight gain, changes in dietary habits, and VPA dosage have failed to reveal predictive factors for VPA-induced weight gain. In attempting to generate animal models of VPA-induced weight gain, we and others have demonstrated that VPA does not cause weight gain in mice (9) or rats (10, 11); however, VPA can induce a significant increase in body weight in female rhesus monkeys (12), suggesting that this side effect may be a characteristic of primate physiology. There is currently no clear mechanism to explain how VPA causes weight gain.

VPA can inhibit both class I and II HDACs, with a high potency for class I HDACs (4). The role of HDAC activity in adipocyte differentiation is not well defined; however, recent work has highlighted a requirement for reduced HDAC activity for transcriptional activation of adipogenic genes *in vitro* (13). We demonstrate that VPA treatment inhibits mouse 3T3-L1 and human adipocyte differentiation. Adipocytes differentiate from preadipocytes via a well defined series of transcriptional events involving several key transcription factors, including members of the CCAAT/enhancer-binding protein (C/EBP) family, peroxisome proliferator-activated receptor γ (PPARγ), and steroid regulatory element-binding protein (SREBP) (14). Inhibition of differentiation by VPA is characterized by diminished PPARγ and SREBP1a mRNA and protein. The mRNA levels for C/EBPα were not affected by VPA treatment; however, there was a significant reduction in C/EBPα protein levels. Experiments using either the structurally unrelated HDAC inhibitor trichostatin A (TSA) or valpromide (VPM), an amide analog of VPA that does not inhibit HDACs, support the hypothesis that HDAC activity is required for initiation of adipogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—Mouse 3T3-L1 cells were obtained from the American Type Culture Collection and subcultured in 5% CO_2 at 37 °C. The cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen), with 10% heat-inactivated calf serum (Invitrogen) and penicillin G/streptomycin sulfate (Invitrogen). The medium was changed every 2 days, and the preadipocytes were maintained at <50% confluence.

For 3T3-L1 differentiation experiments, 2 days after preadipocytes reached confluence they were treated with medium to induce differen-

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[1] The abbreviations used are: VPA, valproic acid; C/EBP, CCAAT/enhancer-binding proteins; PPARγ, peroxisome proliferator-activated receptor γ; SREBP, steroid regulatory element-binding protein; HDAC, histone deacetylase; TSA, trichostatin A; VPM, valpromide; TAG, triacylglycerol; MDI, differentiation inducing medium; ROS, rosiglitazone; TGZ, troglitazone; LBD, ligand-binding domain; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; 4-PB, 4-phenylbutyrate.

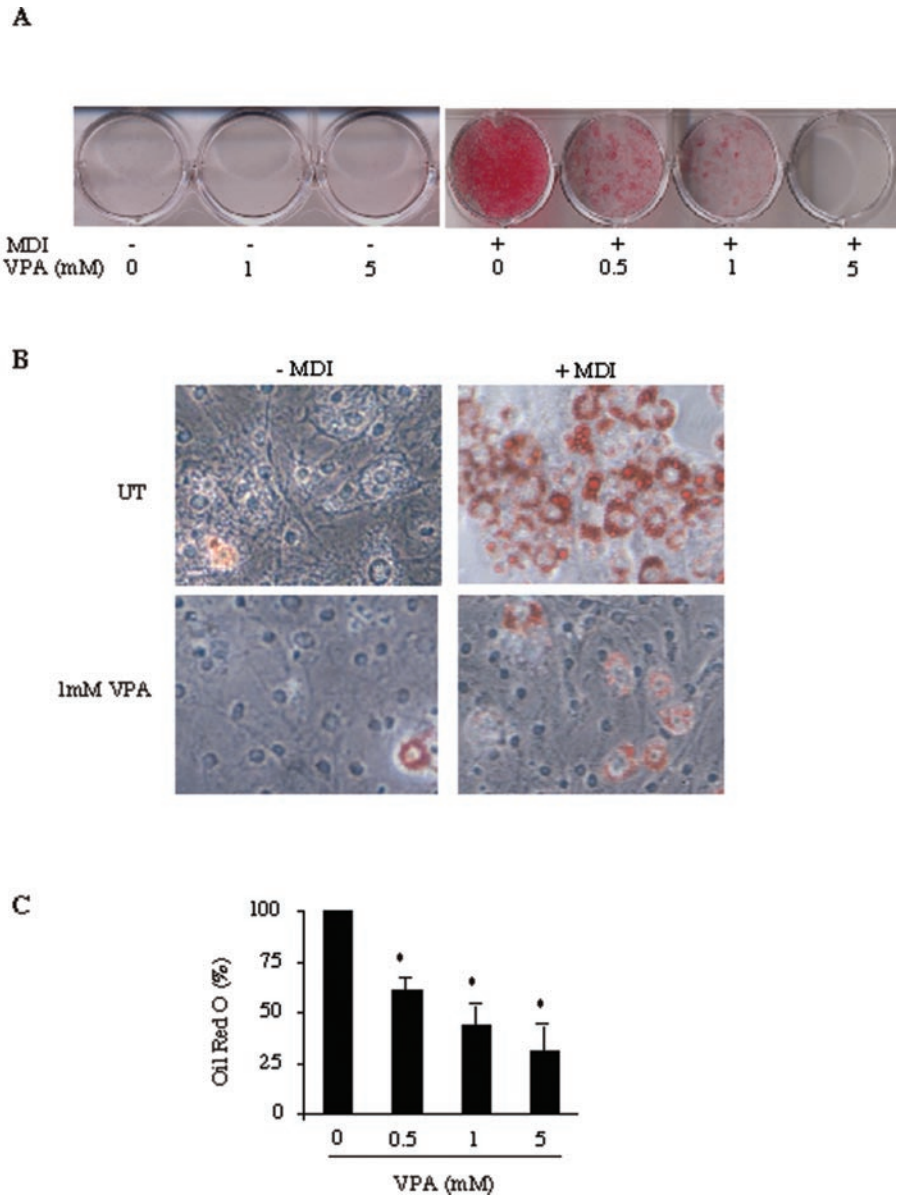

FIG. 1. **VPA inhibits differentiation of mouse 3T3-L1 preadipocytes.** *A*, visualization of triacylglycerol levels by Oil Red O staining 13 days after initiation of differentiation. The cells were grown in the presence (+) or absence (−) of MDI medium (3-isobutyl-1-methylxanthine, dexamethasone, and insulin) for the first 2 days and treated daily with VPA (0–5 mM). In the absence of MDI, untreated or VPA-treated cells have similar Oil Red O staining. In the presence of MDI, VPA dose-dependently reduces adipocyte differentiation. *B*, high magnification (200×) picture of cells cultured using the same conditions as in *A*, demonstrating that few adipocytes form in the absence of MDI whether untreated (*UT*) or treated (1 mM VPA). MDI induces adipocyte formation, which is reduced by VPA treatment. *C*, quantification of Oil Red O staining of cells cultured in MDI confirmed that VPA treatment significantly reduced formation of adipocytes. The data represent the mean percentage levels compared with untreated (set at 100% differentiation). *, $p < 0.001$.

tiation (MDI: DMEM, 10% fetal bovine serum (FBS; CanSera), 250 nmol/liter dexamethasone (Sigma), 0.5 nmol/liter 3-isobutyl-1-methylxanthine (Sigma), and 100 nmol/liter (0.58 μg/ml) human insulin (Roche Applied Science)). After 2 days in MDI, the preadipocytes were cultured in DMEM containing 10% FBS and 5 μg/ml insulin. Subsequent medium changes occurred every second day. VPA and 4-phenylbutyrate (4-PB) were dissolved in phosphate-buffered saline, whereas VPM and TSA were dissolved in Me_2SO and applied as indicated in figure legends. Me_2SO levels were kept under 0.1%. For PPARγ agonist-induced differentiation, preadipocytes (2 days post-confluent) were treated with DMEM, 10% FBS, 100 nmol/liter insulin, and a PPARγ agonist (troglitazone (TGZ; 5 nM or 10 nM) or rosiglitazone (ROS; BRL49653, 250 nM or 1 μM)). After 2 days the medium was changed to DMEM, 10% FBS, 5 μg/ml insulin, and the PPARγ agonist. Subsequent medium changes occurred every 2 days with medium containing the PPARγ agonist.

Human adipocyte experiments were conducted by Zen-Bio Inc. (Contract DAL040403). Primary human preadipocytes were obtained from patients undergoing liposuction surgery. Two lots of cells were used including those from an individual (L091901, male, 48 years old, body mass index of 25.07) and a mixed lot (SL0023, six female individuals, average age of 48, average body mass index of 26.07). Details for preadipocyte differentiation can be found at www.zen-bio.com. For each experiment, a control consisting of a triplicate set of cells treated with 5 ng/ml tumor necrosis factor α was included. The addition of ROS, Zen-Bio PPARγ agonist, or tumor necrosis factor α was included with each medium change.

Oil Red O Staining—The cells were stained with Oil Red O and quantified as previously described by Kasturi and Joshi (15). Stain bound to lipid droplets was extracted with isopropanol, and the absorbance of the dye-triglyceride complex was measured at 520 nm.

[^{3}H]Thymidine Incorporation—The cells were pulsed labeled with [^{3}H] thymidine (2 μCi/ml; Amersham Biosciences) at 37 °C for 1 h prior to harvesting (1, 10, 20, or 30 h after the induction of differentiation), similar to the method of Tang and Lane (16). Briefly, the cells were placed on ice and rinsed twice with cold phosphate-buffered saline. To precipitate the DNA, the cells were incubated with 10% trichloroacetic acid for 1 h at 4 °C. The cells were then washed with absolute alcohol at room temperature and allowed to air dry at room temperature for 2 h. DNA was extracted using 0.1 M NaOH for at least 1 h at room temperature, and radioactivity was counted in acidified scintillation fluid.

Analysis of Lipid Synthesis—The cells were pulse labeled with [^{14}C] acetic acid (250 nCi/ml; Amersham Biosciences) for 2 h at 37 °C. [^{14}C] acetic acid will be metabolized and incorporated into acetyl CoA, which is subsequently incorporated into newly synthesized triacylglycerol. The cells were rinsed twice with cold phosphate-buffered saline and scraped into 1 ml of methanol:water (5:4, v/v) and sonicated. The organic and aqueous phases were extracted using chloroform:methanol (1:2, v/v) with 0.58% NaCl. The organic phase was washed three times with ideal upper phase buffer (0.57% methanol:NaCl:choloroform, 45:47:3, v/v/v), evaporated, and then resuspended in chloroform. Radiolabeled lipids were resolved by TLC in petroleum ether:diethyl ether:acetic acid (60:40:1, v/v/v). Standards were identified using iodine staining, and the TLC plates were exposed to film. Radiolabeled lipids

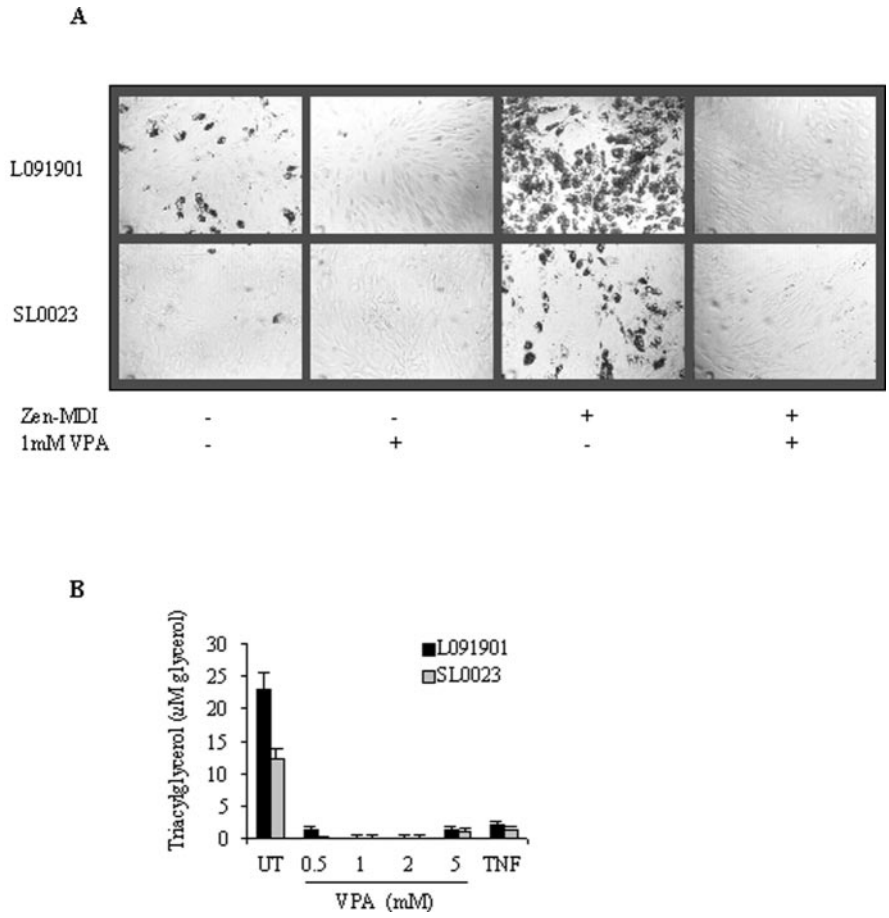

FIG. 2. **VPA inhibits differentiation of human preadipocytes.** *A*, representative photomicrographs of two lots of subcutaneous preadipocytes from one individual (L091901) and cells pooled from six individuals (SL0023) stained with Oil Red O 12 days after initiation of differentiation in the presence or absence of Zen-Bio's differentiation medium (Zen-MID) and/or daily treatment with 1 mM VPA. VPA does not induce adipocyte formation in absence of Zen-MID and inhibited adipocyte formation in the presence of Zen-MID. *B*, quantification of TAG levels from cells described in *A* that were treated daily with VPA (0–5 mM) or 4 ng/ml of tumor necrosis factor α (*TNF*). The data represent the means from one experiment performed in triplicate. *UT*, untreated.

identified by co-migration with authentic standards were scraped into vials and quantified by scintillation counting.

Northern Analysis—RNA was isolated using TRIzol reagent (Invitrogen). Twenty micrograms of total RNA was separated on a 1.5% agarose, 0.67% formaldehyde gel and transferred to BrightStar Plus membrane (Ambion Inc.). The mouse PPARγ, C/EBPα, and SREBP1a cDNAs were labeled with [α-^{32}P]dATP (2 μCi/ml) using the Strip-EZ DNA probe synthesis and removal kit (Ambion). The blots were incubated with probe (1×10^6 cpm/ml) in ULTRAhyb buffer (Ambion) overnight and washed, and the signals were visualized by autoradiography and quantified using a Molecular Dynamics Storm PhosphorImager.

Western Analysis—The cells were washed two times in ice-cold phosphate-buffered saline, and the extracts were isolated using high salt lysis buffer (10 mM Tris-Cl, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 1× complete protease inhibitors (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride), sonicated, clarified by centrifugation (10 min at 15 000 × *g*), and quantified by Bradford analysis (Bio-Rad). For SREBP1a analysis, the cells were treated for 4 h before harvesting and again during lysis with 25 μg/ml of the proteasome inhibitor, *N*-acetyl-leucine-leucine-norleucinal (Sigma). Twenty to forty micrograms of protein/lane were used for Western analysis. The proteins were detected with antibodies from Santa Cruz Biotechnology Inc. including anti-PPARγ (E-8), anti-SREBP1a (2A4), anti-C/EBPα (14AA), or rabbit polyclonal anti-actin (Sigma). After washing in Tris-buffered saline, the blots were incubated with sheep anti-rabbit or sheep anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Chemicon International). Protein expression was visualized with enhanced chemiluminescence (PerkinElmer Life Sciences) and signal-quantified using NIH Image software. Western blots were stripped in stripping solution (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 15 min at 50 °C, washed, blocked, and reprobed.

Transient Transfection and PPAR-LBD Assay—Reporter gene assays were completed in HepG2 cells maintained in DMEM with 10% FBS. The cells were plated in 24-well plates at 7.5×10^4 cells/well the day prior to transfection. The cells were transfected in triplicate with the mammalian transfection mixture of 200 ng of reporter plasmid FR-luc, 100 ng of internal reference plasmid pCMV β-galactosidase, and 12.5 ng of either the GAL4 DNA-binding domain expression plasmid (BD-Gal4) or the expression plasmid for GAL4 DNA-binding domain fused with the PPARγ ligand-binding domain (PPAR-Gal4) using FuGENE 6 (Roche Applied Science). The subsequent day, the cells were treated overnight with compounds. Twenty hours after the addition of drugs, the cells were harvested, and the luciferase activity was determined using the Enhanced Luciferase assay kit (BD Pharmingen). The results were normalized using β-galactosidase activity and represent the mean data from three independent experiments.

RESULTS

VPA Inhibits Adipocyte Differentiation—To determine whether VPA could induce adipocyte differentiation, mouse 3T3-L1 cells were cultured for 2 days in DMEM containing 10% FBS, insulin, and VPA (1 or 5 mM). After 13 days in culture, there were very few cells staining red with Oil Red O, which binds to triacylglycerol (TAG) in fat droplets, a hallmark of adipocyte formation (Fig. 1*A*, *left panel*; for a higher magnification, see Fig. 1*B*). As a positive control, the cells induced to differentiate in medium consisting of DMEM containing 10% FBS, 3-isobutyl-1-methylxanthine, dexamethasone (0.25 μM), and insulin (MDI) but no VPA, contained numerous lipid droplets (Fig. 1*A*). These data show that chronic VPA treatment was unable to induce differentiation of 3T3-L1 cells into adipocytes.

We next examined the effect of VPA treatment on MDI-induced preadipocyte differentiation. Daily treatment with VPA significantly inhibited MDI-induced adipocyte differentiation in a dose-dependent manner; shown by the decrease in lipid content measured 13 days after the initiation of differentiation (Fig. 1, *A* and *C*). Under higher magnification, almost 100% of MDI-treated cells displayed lipid droplet formation, whereas in the presence of MDI and VPA, there was a reduction in the number of cells that had lipid droplet formation (Fig. 1*B*, *right panels*). To quantify the decrease in TAG levels fol-

lowing daily treatment with VPA, the cells were metabolically labeled with [^{14}C]acetic acid at day 13 post-differentiation in the presence or absence of VPA. Chronic VPA treatment resulted in an average significant decrease in TAG synthesis by 51% (untreated 21,723 ± 4,782 dpm/mg protein *versus* 1 mM VPA 10,698 ± 2,855 dpm/mg protein, $p < 0.001$).

In addition to working with 3T3-L1 cells, Zen-Bio Inc. was contracted to conduct a double-blind study to examine the effects of VPA on differentiation of primary human subcutaneous preadipocyte cultures *in vitro*. Similar to the studies in 3T3-L1 cells, the effect of VPA on adipocyte differentiation was measured both in the absence and presence of differentiation inducing media (Zen-DIM). These studies were conducted on two cell lots, one from a single individual (L091901) and the other from a pooled sample containing adipocytes from six individuals (SL0023). In both lots VPA by itself did not induce differentiation of human preadipocytes, rather it inhibited differentiation in the presence of Zen-DIM (Fig. 2*A*). Lot L091901 exhibited some differentiation in the absence of Zen-DIM, which was also inhibited by VPA treatment (Fig. 2*A*). Quantification of TAG levels revealed that VPA inhibited differentiation in a dose-dependent manner more effectively than tumor necrosis factor α, a known inhibitor of preadipocyte differentiation (Fig. 2*B*). Collectively these data show that in both mouse and human cells chronic VPA treatment inhibits adipocyte differentiation.

We then examined whether the inhibitory effects of VPA on 3T3-L1 differentiation occurred following a single treatment with VPA. When 1 mM VPA was added once concurrent with MDI at the onset of differentiation, VPA reduced adipocyte formation (Fig. 3*A*). This finding suggested that VPA treatment might affect critical steps that occur at the onset of differentiation. After reaching confluence preadipocytes become quiescent; however, in response to differentiation media they undergo mitotic clonal expansion. Because inhibition of mitotic clonal expansion can inhibit differentiation we measured whether VPA affected re-entry into the cell cycle. Measuring [^{3}H]thymidine incorporation into cellular DNA, differentiating preadipocytes exhibited a well characterized entry into and exit from S phase of the cell cycle at ~12–16 h following addition of the MDI (Fig. 3*B*). In the presence of VPA, this curve remains unchanged, indicating that VPA does not affect mitotic clonal expansion. Daily VPA treatment beginning after the removal of the MDI (days 3–13) caused the inhibition of differentiation to the same extent as VPA treated concurrently with the MDI (day 0–2) (Fig. 3*C*). Daily VPA treatment beginning 7 days after the initiation of differentiation (day 7–13) also reduced Oil Red O levels; however, this reduction was not significant compared with untreated cells. These results suggest that VPA is affecting critical events that occur both at the onset of differentiation, as well as events required during the initial maturation of the adipocytes.

VPA Inhibits PPARγ-induced Differentiation—PPARγ agonists induce differentiation of adipocytes, whereas PPARγ antagonists inhibit differentiation (17). The PPARγ ligands TGZ and the more potent ROS were used to induce the differentiation of 3T3-L1 and human preadipocytes. When co-treated with VPA and either TGZ or ROS, differentiation of 3T3-L1 cells was inhibited by 60 and 43%, respectively (Fig. 4*A*), suggesting that VPA can inhibit differentiation mediated by PPARγ. In support of these findings, VPA was also able to inhibit ROS-induced differentiation of human preadipocytes, assessed by TAG levels (Fig. 4*B*). Furthermore, VPA inhibited differentiation of human preadipocytes when differentiation was induced with 10 μM

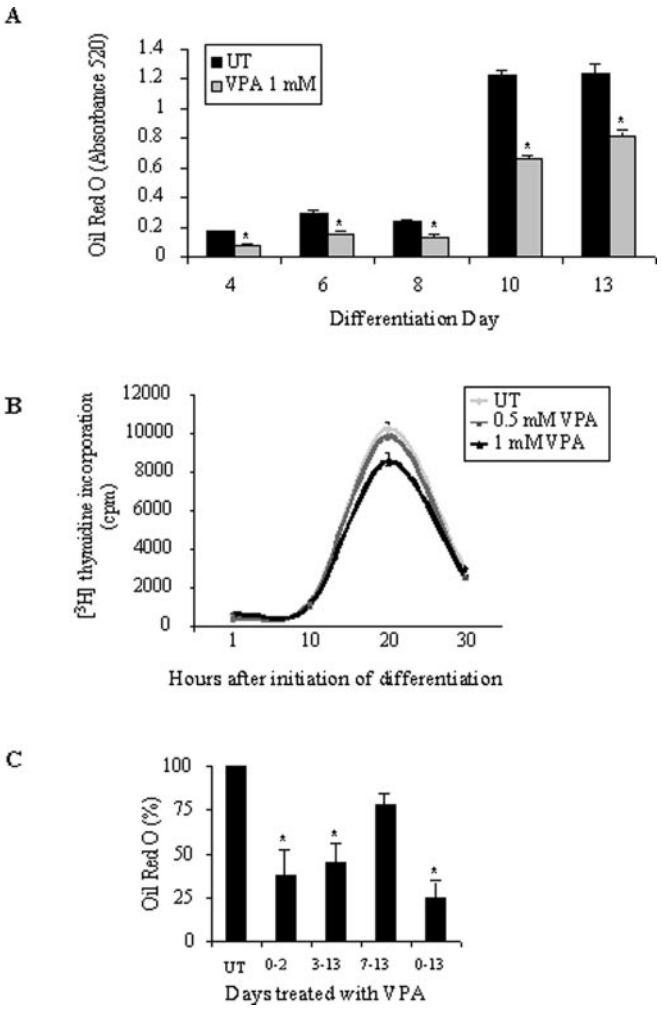

FIG. 3. **Temporal effects of VPA treatment on adipocyte differentiation.** *A*, 3T3-L1 cells treated once at day 0 with 1 mM VPA have significantly lower amounts of Oil Red O staining compared with untreated (*UT*) cells. The data represent the mean levels of Oil Red O from one experiment performed in triplicate; similar findings were obtained in three independent experiments. *B*, [^{3}H]thymidine incorporation into cellular DNA measured at 1, 10, 20, and 30 h following the addition of MDI, demonstrating no significant difference in clonal expansion in the absence (*UT*) or presence of VPA. Representative data from one experiment performed in triplicate are shown; similar data was obtained in two separate experiments. *C*, quantification of Oil Red O staining of cells differentiated with MDI (days 0–2), untreated (*UT*), treated daily with VPA (days 0–2, 3–13, 7–13, or 0–13). The data represent the mean percentage levels compared with untreated (set at 100% differentiation). *, $p < 0.001$

Zen-Bio proprietary non-thiazolidinedione PPARγ agonist.[2] These data show that VPA inhibits PPARγ-induced differentiation in mouse and human preadipocytes.

VPA Is Not a PPARγ Ligand—Because of the ability of VPA to block PPARγ-induced differentiation, we conducted reporter assays to assess whether VPA interacted with the LBD of PPARγ. Because TGZ and ROS had equivalent activities, these experiments were conducted using TGZ. VPA induced a weak activation of the PPARγ-LBD chimeric construct (PPAR-Gal4), compared with a greater than 40-fold increase by TGZ (Fig. 4*C*). The VPA-induced fold increase in PPAR-Gal4 was similar to the increase in activation of the control plasmid (BD-Gal4) lacking the PPARγ-LBD. These results suggest that VPA may nonspecifically increase the activation of PPAR-Gal4. VPA increases the activity of many reporter genes in transient trans-

[2] D. C. Lagace and M. W. Nachtigal, unpublished observations.

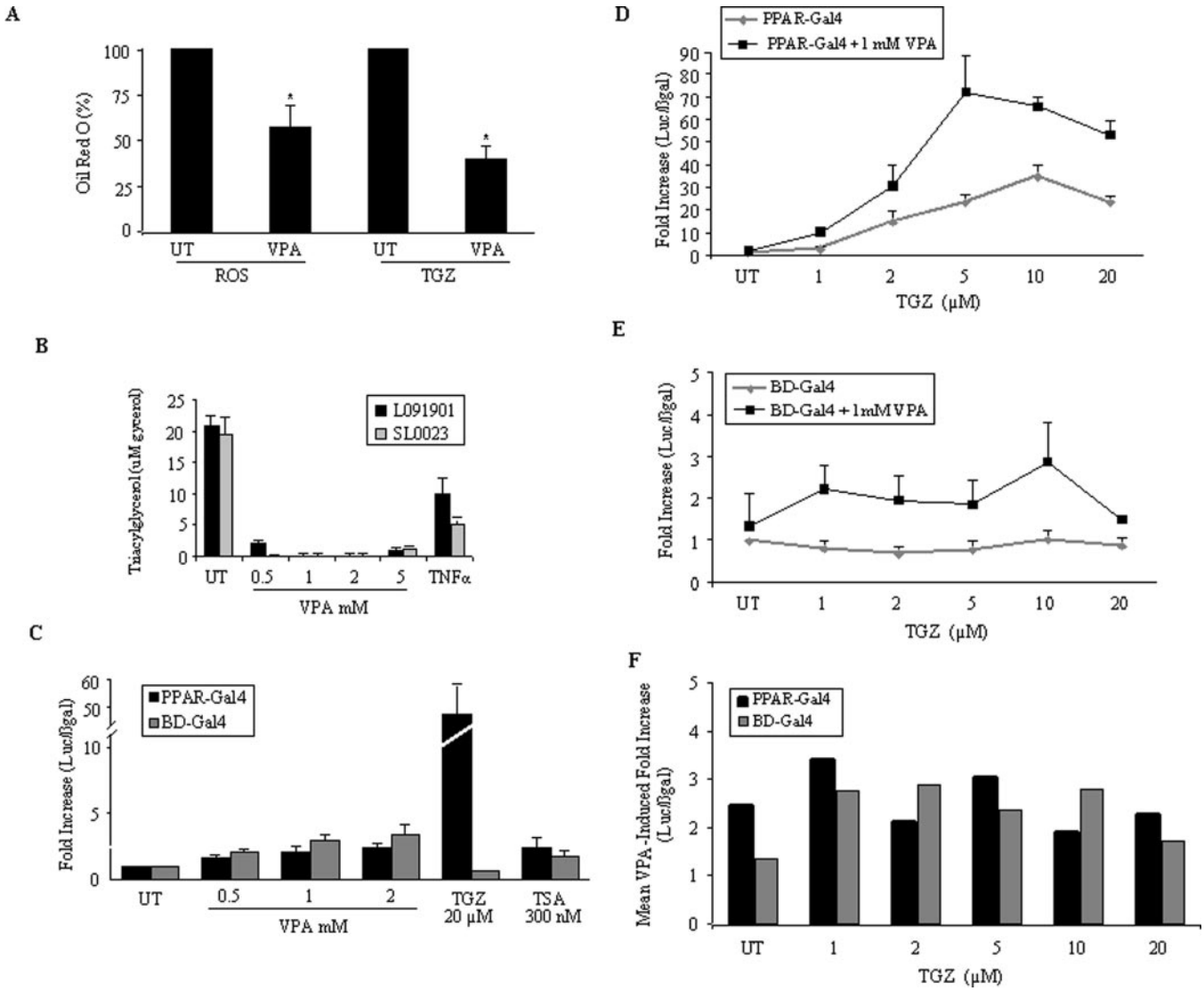

FIG. 4. **VPA inhibits PPARγ-induced differentiation in mouse and human preadipocytes but does not act as a PPARγligand.** *A*, quantification of Oil Red O staining of 3T3-L1 cells differentiated in the presence of a PPARγ agonist, 1 μM ROS, or 10 nM TGZ, demonstrating significant reduction in staining when cells were co-treated daily with VPA on days 0–13. The data represent the mean percentage levels compared with untreated (*UT*, set at 100% differentiation). *, $p < 0.001$. *B*, quantification of triacylglycerol levels from two lots of human preadipocytes, demonstrating cells differentiated with 1 μM ROS had more triacylglycerol than cells that were co-treated daily with VPA (0–5 mM). Cells treated with 4 ng/ml of tumor necrosis factor α at each medium change are shown. The data represent the mean values from one experiment performed in triplicate. *C*, fold induction in luciferase activity in the presence of the Gal4 expression plasmid (BD-Gal4) or the Gal4 expression plasmid with the PPARγ ligand-binding domain (PPAR-Gal4) following treatment with either VPA (0.5–2 mM), TGZ (20 μM), or TSA (300 nM) in HepG2 cells. *D*, dose-response curve (1–20 μM) for TGZ-induced increase of PPAR-Gal4 activity in the absence or presence of 1 mM VPA. *E*, dose response (1–20 μM) for TGZ on the BD-Gal4 activity in the absence or presence of 1 mM VPA. *F*, summary of VPA-induced fold increase from experiments described in *D* and *E*, demonstrating that the differences in activation of BD-Gal4 *versus* PPAR-Gal4 induced by VPA are not significant. The data shown in *B–F* are normalized with β-gal activity; similar findings were obtained in three independent experiments.

fection experiments and is suggested to be due to VPA acting as a HDAC inhibitor (4). We found that the HDAC inhibitor TSA induces a similar fold increase as VPA in BD-Gal4 and PPAR-Gal4 reporter activity (Fig. 4*C*). These data suggest that non-specific activation of reporter genes may be due to an indirect effect of HDAC inhibitors.

PPARγ partial agonists or antagonists can inhibit 3T3-L1 differentiation. Because VPA inhibits 3T3-L1 differentiation, we examined the effect of VPA on the dose-response curve for TGZ-induced PPARγ activity to determine whether VPA may be acting as a PPARγ partial agonist or antagonist. TGZ dose-dependently induced PPARγ activity with a mean fold induction of luciferase activity ranging from 23 ± 2.5 to 35 ± 5.2 (Fig. 4*D*). If VPA was acting as a partial agonist or antagonist, it should inhibit TGZ activity. VPA induced a mean 2.5 ± 0.23-fold increase (Fig. 4*F*) in TGZ-induced PPARγ activity (range, 53 ± 6.3 to 72 ± 16.0 (Fig. 4*D*)); however, this fold increase by VPA was similar to the increase in BD-Gal4 reporter activity (Fig. 4, *E* and *F*). Unlike VPA, TGZ did not induce the control BD-Gal4 reporter. TSA had similar effects as VPA in inducing an ~2-fold increase in TGZ-activated PPARγ and BD-Gal4 reporter activity.[2] These data imply that VPA (0.5–2 mM) does not act as a PPARγ partial agonist or antagonist, most likely producing its effects through its HDAC inhibitory activity.

VPA Treatment Inhibits PPARγ and SREBP1a mRNA Levels—The onset of differentiation in adipocytes involves activation of gene expression including C/EBPα/β/δ, PPARγ, and SREBP1a. Northern analysis was conducted to determine whether the reduction in differentiation by VPA was accompanied by changes in gene expression. Prior to the onset of differentiation, preadipocytes have undetectable levels of C/EBPα, PPARγ, and SREBP1a mRNA (Fig. 5*A*). Twenty-four

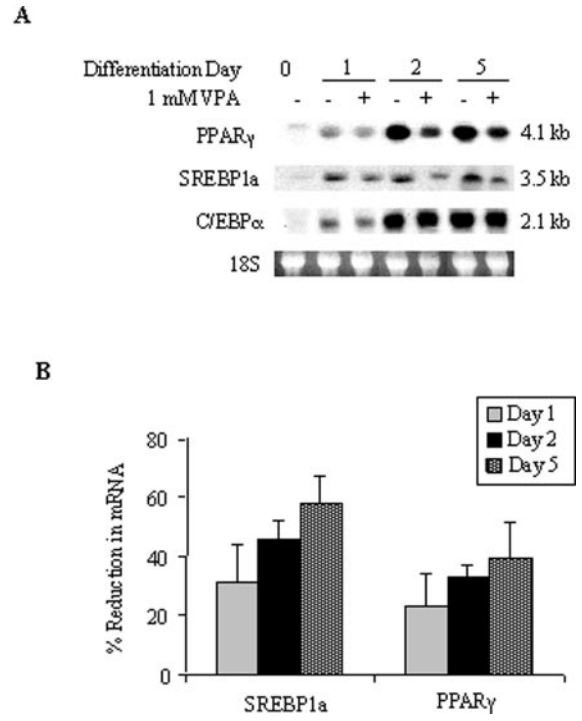

FIG. 5. **VPA reduces SREBP1a and PPARγ, but not C/EBPα, mRNA.** *A*, Northern analysis of mRNA from 3T3-L1 cells prior to the addition of MDI medium (day 0) and days 1, 2, and 5 after addition of MDI in the absence or presence of 1 mM VPA. *B*, mean percentage reduction in mRNA levels for SREBP1a and PPARγ comparing 1 mM VPA treated to untreated cells from two independent experiments, performed in duplicate at each time point.

hours following the initiation of differentiation, a weak signal for the three transcripts was detectable. At days 1, 2, and 5 following the initiation of differentiation, VPA-treated cells had reduced levels of PPARγ and SREBP1a mRNA, and no change in C/EBPα mRNA levels compared with control cells (Fig. 5, *A* and *B*). We found that VPA reduced glyceraldehyde-3-phosphate dehydrogenase mRNA levels at days 2 and 5 by 33 ± 6 and 29 ± 8%, respectively, and therefore could not use it to normalize for equal loading. Because there was no change in C/EBPα levels at days 2 and 5 following treatment, it is unlikely that the reduction in PPARγ and SREBP1a gene expression is due to unequal loading; confirmed by 18 S rRNA loading (Fig. 5*A*).

VPA Treatment Reduces PPARγ, SREBP1a, and C/EBPα Protein Levels—To determine whether VPA-induced a concomitant reduction in mRNA and protein levels for PPARγ, total cell protein lysates were obtained from cells treated with VPA 1, 3, and 5 days after addition of the MDI medium. PPARγ has two isoforms ($PPAR\gamma_1$ and $PPAR\gamma_2$) that are undetectable in preadipocytes (day 0), are expressed at low levels 1 day following initiation of differentiation (day 1), and are clearly detectable at all time points tested after day 3 (Fig. 6*A*). VPA treatment caused a reduction in $PPAR\gamma_1$ and $PPAR\gamma_2$, with a maximum reduction greater than 50% on day 7 (Fig. 6*C*). After detection of PPARγ, the blots were stripped and incubated with an antibody against C/EBPα, which detects both the 42- and 30-kDa alternative translation products. Although C/EBPα proteins were undetected in preadipocytes, a signal was detected at day 1 of differentiation and increased throughout differentiation, days 3–5 (Fig. 6*A*). Compared with untreated cells, daily VPA treatment reduced the amount of p42 and p30 C/EBPα protein (Fig. 6*C*), which was surprising because no changes in C/EBPα mRNA levels were detected in the presence of VPA.

The precursor (P; 125 kDa) and the cleaved (C; 68 kDa) forms of SREBP1a were detected in differentiating adipocytes (Fig. 6*B*). In comparison with the expression of the SREBP1a(P), which appears 1 day after inducing differentiation, SREBP1a(C) was detected at later time points, with higher amounts of expression at day 4 and 7. Daily VPA treatment did not have an effect on the amount of SREBP1a(P) but consistently decreased the SREBP1a(C) at day 4 and later time points (Fig. 6, *B* and *C*).

HDAC Inhibition and Adipogenesis—We sought to determine whether other HDAC inhibitors had similar effects on adipogenesis and confirm whether VPA treatment in our model was associated with HDAC inhibition. Similar to VPA, daily TSA (3 nM) or 4-PB (1.5 mM) treatment inhibited 3T3-L1 differentiation (Fig. 7, *A* and *B*). The dose of VPA, TSA, and 4-PB was chosen based on the known HDAC IC_{50} for these compounds (3, 4, 18–20). For hydroxamic acid compounds such as TSA the *in vitro* HDAC IC_{50} is within the nM-μM range, whereas short chain fatty acids such as VPA and 4-PB have HDAC IC_{50} in the mM range (21, 22). Because the effect of TSA and 4-PB were similar, future experiments were conducted using TSA, a more potent and commonly used HDAC inhibitor. Similar to VPA, TSA caused a dose-dependent inhibition of adipocyte differentiation (Fig. 7*C*). In comparison with VPA, VPM (1 mM), an amide analog of VPA that does not inhibit HDAC activity, did not significantly affect 3T3-L1 differentiation (Fig. 7, *A* and *B*).

To examine the HDAC inhibitory activity of VPA, we treated 3T3-L1 cells with VPA (1 mM), TSA (3 nM), or VPM (1 mM) daily and assessed histone acetylation at days 3 and 7 following initiation of differentiation. Compared with untreated cells, VPA and TSA induced histone H3 acetylation (Fig. 7*D*). Some histone H3 acetylation was observed in VPM-treated cells, but the levels were consistently less than that produced by VPA or TSA (Fig. 7*D*).

We determined whether the reduction in adipocyte differentiation by TSA affected C/EBPα, PPARγ, and SREBP1a protein and mRNA expression. TSA reduced $PPAR\gamma_1$, $PPAR\gamma_2$, p42 and p30 C/EBPα, and the cleaved form of SREBP1a(C) but not SREBP1a(P) (Fig. 8, *A* and *B*). In contrast to the effects of VPA or TSA, VPM treatment, which does not inhibit differentiation, did not affect protein expression (Fig. 8*A*). Similar to VPA-treated cells, TSA treatment caused reduced levels of PPARγ (average reduction, 23 ± 7%) and SREBP1a (average reduction, 18 ± 11%) mRNA levels (Fig. 8*C*). Unlike VPA treatment, which had no effect on C/EBPα mRNA levels, TSA treatment reduces C/EBPα mRNA levels (average reduction, 37 ± 5%), and VPM had no effect on mRNA levels compared with untreated cells (Fig. 8*C*).

DISCUSSION

Our results show that VPA treatment prevents mouse and human adipocyte differentiation *in vitro*. Pharmacologic manipulation of HDAC activity with TSA and 4-PB inhibited preadipocyte differentiation, whereas VPM did not, leading to the notion that the HDAC inhibitory properties of VPA may be responsible for blocking adipogenesis. TSA treatment reduced mRNA levels for PPARγ, C/EBPα, and SREBP1a, whereas VPA treatment reduced PPARγ and SREBP1a mRNA but not C/EBPα mRNA levels. Inhibition of differentiation by VPA and TSA was accompanied by a reduction in TAG and decreased protein levels for PPARγ, C/EBPα, and SREBP1a. Based on these results, we hypothesize that HDAC activity is required for adipocyte differentiation.

The role of HDACs during adipocyte differentiation remains largely unknown compared with their role in the differentiation of other cell types such as skeletal muscle (21). In models

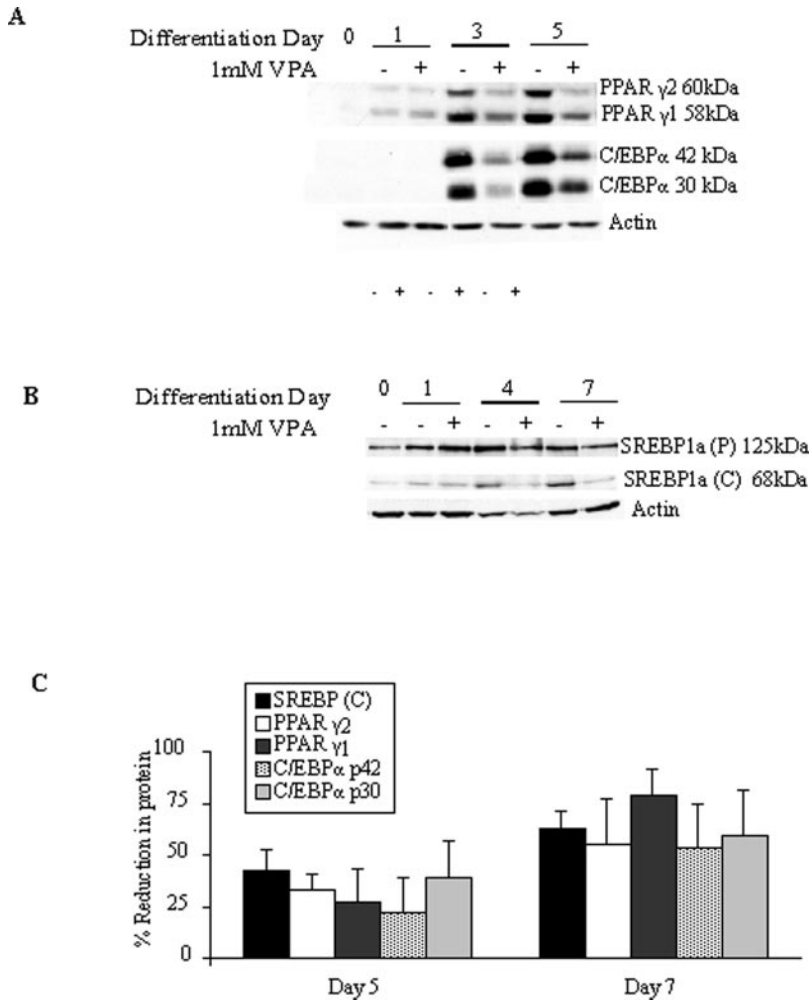

FIG. 6. **VPA treatment reduces PPAR$\gamma_1$and PPARγ_2, p30 and p42 C/EBPα, and SREBP1a(C) protein.** *A*, Western analysis of whole cell protein extracts from 3T3-L1 cells obtained prior to the addition of MDI medium (day 0) and days 1, 3, and 5 after the addition of MDI in the absence or presence of 1 mM VPA. *B*, Western analysis of whole cell protein extracts from 3T3-L1 cells prior to (day 0) or after the addition of MDI medium (days 1, 4, and 7). Daily VPA (1 mM) treatment reduces protein levels for mature SREBP1a(C) but does not affect precursor SREBP1a(P) protein. The cells were pretreated with, and protein samples isolated in the presence of, the proteosome inhibitor *N*-acetyl-leucine-leucine-norleucinal. *C*, mean percentage reduction in mature SREBP1a, PPARγ, and C/EBPα protein levels at days 5 and 7 after initiation of differentiation, comparing VPA (1 mM) treated to untreated cells. The data are representative of two independent experiments performed in triplicate at each time point.

of muscle cell differentiation (C2C12 skeletal muscle cells and human skeletal myoblasts) when HDAC inhibitors were added 1 day prior to the differentiation-inducing medium, they enhanced myogenesis; however, when HDAC inhibitors were added simultaneously to the differentiation medium, myogenesis was inhibited (23). Thus, augmentation or suppression of myogenesis is dependent on the time of HDAC inhibition, suggesting that HDAC activity may provide an important checkpoint to prevent precocious myogenic differentiation. Treatment of 3T3-L1 cells with VPA or TSA 1 day prior to the initiation of differentiation produces no abrogation in adipogenesis.[2] Our results show that HDAC inhibition with VPA or TSA causes a dose-dependent attenuation of adipogenesis when added once at the initiation of differentiation or chronically throughout the differentiation period. Two recent studies have examined the effect of a single dose of VPA on adipogenesis in the presence of differentiation medium (MDI). Fajas *et al.* (13) report that treating 3T3-L1 cells once with VPA (1.5 mM) at the onset of treatment with MDI caused a significant induction of adipocyte differentiation. By contrast Wiper-Bergeron *et al.* (25) illustrate that a single treatment with VPA (10 mM) at the onset of treatment with MDI did not affect differentiation. Repeating these experiments using the protocols described in these manuscripts, we found that VPA did not promote and always inhibited adipogenesis.[2]

Which of the numerous HDACs may be important for adipogenesis? TSA, like most HDAC inhibitors, equally inhibits all known HDACs in a reversible fashion by displacing the requisite zinc ion within the active site (21). Unlike other known HDAC inhibitors, VPA inhibits class I HDACs with five times greater potency than class II HDACs *in vitro* and *in vivo* (4). In our experiments we tested VPA at doses of 0.5–5 mM and show that inhibition was dose-dependent. Based on the published IC_{50} for VPA (class I (HDAC2) IC_{50} = 0.54 mM; class II (HDAC5) IC_{50} = 2.8 mM (4)), the lower doses of VPA used in our experiments to inhibit differentiation would inhibit class I HDACs and may not significantly affect class II HDACs. This suggests that class I HDACs may be critical for adipogenesis. Moreover, it is unlikely that the proteosome-dependent degradation of HDAC2 induced by VPA, but not TSA, is responsible for the similar effects of VPA and TSA to inhibit adipogenesis (24). Wiper-Bergeron *et al.* (25) demonstrated that during the initial 24 h of 3T3-L1 cell differentiation in the presence of dexamethasone, HDAC1 protein levels are reduced by 50%, without affecting HDAC1 mRNA, when compared with cells treated without dexamethasone. We found that HDAC1 protein levels in 3T3-L1 cells were not significantly different between preadipocytes (day 0) and developing adipocytes (days 1, 3, and 5) in the absence or presence of VPA, TSA, or VPM.[2] Although HDACs may remain at a steady state level during adipogenesis, this does not rule out the possibility that they may be dynamically regulated by their localization within the cytoplasm and nucleus. In muscle cell differentiation, the shuttling of HDACs 4, 5, and 7 between the cytoplasm and nucleus plays a critical role in myogenesis (26). To fully explore the role of HDACs in adipogenesis, it will be necessary to identify which HDACs are present in adipocytes and determine whether their localization and activity are dynamically regulated.

Three main classes of transcription factors directly influence fat cell development: PPARγ, C/EBP, and SREBP-1 (27–29).

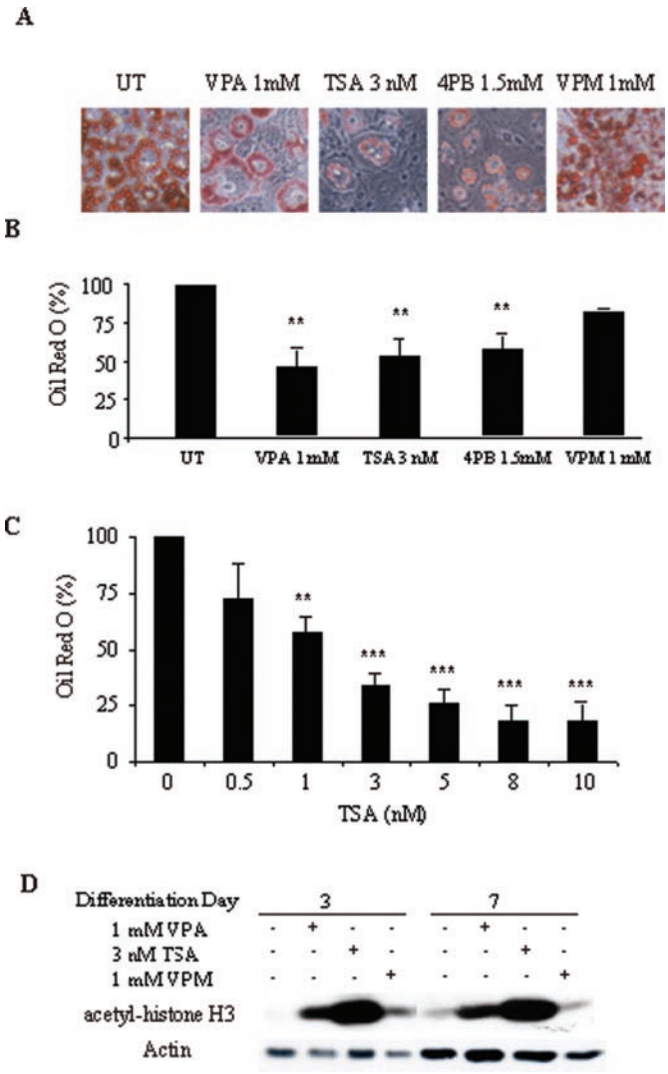

FIG. 7. **HDAC inhibition reduces adipocyte differentiation.** *A*, cells stained with Oil Red O 13 days after initiation of differentiation in MDI. In comparison to untreated (*UT*) cells daily treatment with the HDAC inhibitors VPA (1 mM), TSA (3 nM), or 4PB (1.5 mM) inhibited differentiation, whereas VPM (1 mM), which does not inhibit HDACs, does not affect adipocyte differentiation. *B*, quantification of Oil Red O staining of cells cultured using the same conditions as *A*; TSA, 4PB, and VPA reduced formation of adipocytes, whereas there was no significant difference between untreated (*UT*) and VPM-treated cells. The data represent the mean percentage levels compared with untreated (set at 100% differentiation). *C*, TSA dose-dependently inhibits 3T3-L1 differentiation. The data represent the mean percentage levels compared with untreated (set at 100% differentiation). *D*, Western analysis of 3T3-L1 cells obtained at days 3 and 7 after the addition of MDI. Daily VPA (1 mM) and TSA (3 nM) treatment induces high levels of acetylated histone H3, compared with untreated and VPM-treated (1 mM) cells. **, $p < 0.01$;***, $p < 0.001$.

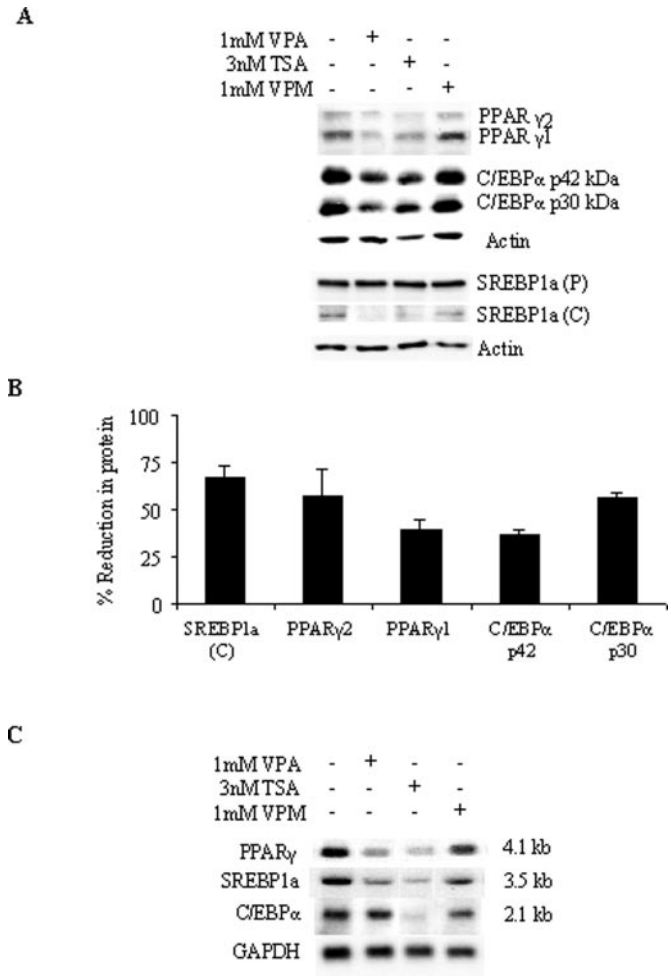

FIG. 8. **VPA and TSA, but not VPM, reduce protein levels for PPARγ, SREBP1a, and C/EBPα and differentially affect mRNA production.** *A*, Western analysis of 3T3-L1 cells obtained 7 days after the addition of MDI. Daily TSA (3 nM) or VPA treatment reduced PPARγ$_1$, PPARγ$_2$, p30 and p42 C/EBPα, and mature SREBP1a(C) protein levels when compared with untreated cells. *B*, mean percentage reduction in protein levels comparing TSA (3 nM) treated to untreated cells from two independent experiments, each performed in duplicate. *C*, Northern analysis of mRNA obtained 5 days after addition of MDI. Similar to VPA, daily TSA (3 nM) treatment reduces mRNA levels for PPARγ, SREBP1a. Unlike VPA treatment, TSA induced a reduction in C/EBPα mRNA levels. VPM does not produce a significant effect on mRNA levels.

The expression of PPARγ, and most importantly PPARγ$_2$, is necessary and sufficient to induce adipogenesis (30). Thiazolidinediones are synthetic PPARγ agonists that can induce differentiation of adipocytes, whereas PPARγ antagonists or partial agonists reduce adipogenesis induced by treatment with either MDI or treatment with thiazolidinediones (31, 32). Chronic treatment of 3T3-L1 preadipocytes with VPA was unable to induce adipogenesis, and VPA blocked adipogenesis induced by either TGZ or ROS, suggesting that VPA does not act as a PPARγ agonist. To test whether VPA can act as PPARγ partial agonist or antagonist, we conducted reporter assays using a PPARγ LBD chimeric receptor (PPAR-Gal4) as the activator of transcription. Lampen *et al.* (33) have shown that VPA (0.5–1.5 mM) can induce activation of a glucocorticoid receptor DNA-binding domain-PPARγ LBD hybrid receptor in Chinese hamster ovary cells. Similarly, using NIH-3T3 cells expressing endogenous PPARγ, Fajas *et al.* (13) demonstrated that VPA (0.5–1.5 mM) can activate a reporter gene (PPRE-TK-luc) driven by PPARγ-binding elements linked to a minimal thymidine kinase promoter. We have shown that VPA (1 mM) enhanced TGZ-induced reporter activation at all doses of TGZ tested (1–20 μM); however, the increase in luciferase activity by PPAR-Gal4 in the presence of VPA is equal to the fold increase in activation of the control protein (GAL4 DNA-binding domain alone, BD-Gal4), supporting the notion that VPA is not specifically activating the PPARγ receptor. Indeed, VPA has been shown to induce a diverse number of promoters including the simian virus-40 (34), cytomegalovirus (3, 35), thymidine kinase,[2] and Rous sarcoma virus (3) promoters. These data suggest that VPA has a nonspecific ability to indirectly induce gene transcription, likely because of its HDAC inhibitory properties (3, 4). We conclude that VPA activation of PPARγ is unlikely to be due to a direct effect of VPA interaction with the

PPARγ LBD and that the effects of VPA on adipocyte differentiation are not mediated by VPA binding directly to PPARγ. Furthermore, VPA or TSA inhibition of differentiation is not simply due to prevention of PPARγ expression, because either treatment can inhibit differentiation following the removal of MDI after PPARγ expression has been up-regulated. It remains possible that these drugs may be causing a direct or indirect reduction in PPARγ mRNA transcription or stability.

VPA and TSA caused a reduction in SREBP mRNA and mature protein during adipogenesis, similar to its effects on PPARγ. Surprisingly, the precursor form of SREBP1a protein appears to remain at a steady state following treatment with HDAC inhibitors. In liver cells once the SREBP cleavage-activating protein senses low sterol levels, the precursor SREBP translocates from the endoplasmic reticulum membrane to the Golgi where the mature SREBP is formed by a two-step proteolysis via the Site 1 protease and Site 2 protease (37). In adipocytes it is unknown what signals the maturation of SREBP from its precursor. Inoue *et al.* (38) found that the mRNA levels for SREBP cleavage-activating protein, Site 1 protease, and Site 2 protease in adipocytes remain at a steady state throughout adipogenesis, suggesting that in adipocytes, where sterol levels are not depleted, the mechanism producing the proteolytic activation of SREBP may be unique. Similar to our findings with VPA, others using human immunodeficiency virus protease inhibitors, which also are known inhibitors of adipogenesis, have demonstrated reduced SREBP1a maturation despite steady state precursor levels (39). Future studies are necessary to elucidate the mechanism of SREBP1a processing during adipogenesis and how HDAC inhibitors may affect this process.

It is possible that the down-regulation of PPARγ mRNA by VPA treatment results from VPA-induced down-regulation of C/EBPα protein. Several studies have demonstrated that C/EBPα regulates the expression of PPARγ, most notably, *in vitro* C/EBPα is able to directly bind to the $PPAR\gamma_2$ promoter (40), and *in vivo*, mice with disrupted C/EBPα expression show a reduction in PPARγ levels (41). We see a striking reduction in C/EBPα protein levels with VPA treatment despite no effect on C/EBPα mRNA levels. It remains to be determined whether VPA decreases C/EBPα protein translation or enhances degradation. Because this effect is observed with VPA but not TSA, it is unlikely to be mediated by HDAC inhibition. This suggests that VPA has multiple mechanisms of action to abrogate adipogenesis.

In contrast to our work, others have suggested that HDAC inhibitors promote the differentiation of adipocytes by enhancing the transactivation of PPARγ and C/EBPα (13, 25). Fajas *et al.* (13) demonstrated that treatment with HDAC inhibitors (including VPA) results in dissociation of a PPARγ-Rb-HDAC3 complex, allowing PPARγ transactivation and stimulation of adipocyte differentiation. Similarly, Wiper-Bergeron *et al.* (25) demonstrated that treatment with HDAC inhibitors stimulated adipocyte differentiation by promoting the transcription of C/EBPα by releasing a co-repressor complex comprised of C/EBPβ-mSIN3A-HDAC1 from the C/EBPα promoter. In agreement with these studies, we found that the mRNA and proteins for PPARγ and C/EBPα are made in the presence of VPA and TSA but at greatly reduced levels, which correlates with the limited amount of adipocyte differentiation observed with treatment. It is possible that reduced levels of PPARγ, C/EBP, and SREBP-1 protein are due to decreased production in individual adipocytes or result from the inability of preadipocytes to turn on the differentiation program. Thus, a minimal threshold of active C/EBPα and PPARγ may be required to promote differentiation in individual cells. The molecular mechanism underlying the ability of HDAC inhibitors to regulate C/EBPα and PPARγ expression and activity is unclear based on the current literature. It is likely that regulation of these transcripts is dependent on positive and/or negative factors including co-repressor complexes containing the HDACs. Indeed, it has recently been shown that HDAC interaction with N-CoR can act as positive co-regulators of transcriptional activation (42). Thus, inhibition of HDAC activity may result in blocking transcriptional activity of genes critical for adipocyte differentiation.

We examined VPA effects on adipogenesis with the initial aim of understanding how VPA may induce weight gain in patients. Both fat cell number controlled by preadipocyte proliferation and adipogenesis and fat cell size controlled by lipogenesis contribute to weight gain. We hypothesized that VPA would enhance adipogenesis; however, we found that VPA does the opposite. It is paradoxical that *in vitro* VPA inhibits adipogenesis yet *in vivo* induces weight gain. Weight gain is the outcome of a variety of central and peripheral inputs, and we hypothesize that VPA affects numerous cell types in the central nervous system and periphery. *In vivo*, VPA suppression of adipogenesis may be overcome by compensatory physiological effects with the net outcome of weight gain. Lithium carbonate, another mood stabilizer that induces weight gain *in vivo*, inhibits adipogenesis *in vitro* most likely through its effects on Wnt/GSK3β signaling (43, 44). Unlike lithium carbonate, we suggest that VPA inhibition of adipocyte differentiation is due to its HDAC inhibitory activity. VPA has recently been shown to produce some of its neuronal effects through reduction of inositol biosynthesis, which can be blocked by supplementing cells with inositol. We found that inositol supplementation was unable to reverse the ability of VPA to inhibit adipogenesis.[2] Moreover, VPA is unlikely to mediate its effects via a direct activation of PPARγ. Overall our results highlight a role for HDAC activity in adipogenesis that can be blocked by treatment with VPA.

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