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Induction of GRP78 by valproic acid is dependent upon histone deacetylase inhibition

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Abstract—Valproic (2-propylpentanoic) acid is a commonly used drug in the treatment of bipolar disorder and epilepsy. The molecular mechanism that underlies its clinical efficacy remains controversial and is complicated by the broad range of intracellular effects of valproic acid, including its ability to inhibit histone deacetylase (HDAC) and induce protein chaperone expression. Here we show that an established HDAC inhibitor, trichostatin A, promotes ER chaperone expression in HEK293 cells. Furthermore, we use chemical derivatives of valproic acid to show that the ability to promote GRP78 levels directly correlates with the induction of histone H4 hyperacetylation. These results suggest that exposure to valproic acid enhances chaperone expression by a mechanism that involves histone hyperacetylation.

Valproic (2-propylpentanoic) acid is a small branch-chain fatty acid and a potent and widely prescribed drug that acts both as an anti-convulsant in the treatment of epilepsy and as a mood-stabilizer to control bipolar disorder. Despite its wide use, the specific molecular mechanisms responsible for the clinical effects of valproic acid are not completely understood.[1–3]

The mood stabilizing effects of valproic acid occur at plasma concentrations over 0.35 mM and toxicity can be observed at concentrations over 1.4 mM.[1–4] Exposure to millimolar concentrations of valproic acid can induce a variety of molecular and cellular responses that may be responsible for its clinical efficacy. For example, it has been reported that valproic acid potentiates γ-aminobutyric acid-mediated postsynaptic inhibition,[5] depletes intracellular inositol,[6] inhibits histone deacetylases (HDAC),[7] and can directly[8–10] and indirectly[11] inhibit glycogen synthase kinase (GSK)-3.

Valproic acid has been shown to increase the protein levels of endoplasmic reticulum (ER) chaperones including GRP78, HSP47, calreticulin, protein disulfide isomerase (PDI), as well as the cytosolic chaperone, HSP70.[8,12] Chaperones play an essential role in the folding of nascent proteins. The over-expression of specific chaperones has been shown to confer protection against cellular injury and/or death resulting from a broad array of agents and conditions including cytotoxic chemicals,[13] ER stress,[14] oxidative stress,[15] and ischemia reperfusion.[16]

Here we screen a small library of valproic acid derivatives for their ability to induce the expression of specific chaperones. We specifically investigate the potential role of HDAC inhibition in this effect by monitoring the acetylation of histone H4. Our data show that the ability to induce chaperone expression correlates with increased histone acetylation. These results suggest that valproic acid induces chaperone expression by a mechanism that involves HDAC inhibition.

Valproic acid has previously been shown to inhibit HDAC 1, 2, 5, and 6 at pharmacological concentrations in a variety of cell types including HeLa and

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Neuro2A.[7,17] To investigate the effect of valproic acid in HEK293 cells we exposed cells to 1 mM valproic acid for 0–72 h.[18] Hyperacetylation of histone H4 on lysine 12 was observed in valproic acid treated cells by immunoblot analysis (Fig. 1).[19] Valproic acid treatment resulted in a 3.5-fold increase in histone H4 acetylation within 48 h. A similar increase in histone H4 acetylation was observed when cells were treated with 600 nM trichostatin A (TSA), an established HDAC inhibitor.

We and others have previously reported that valproic acid can induce the expression and accumulation of endoplasmic reticulum (ER) resident chaperones, including GRP78, GRP94, calreticulin, and PDI, as well as the cytoplasmic chaperone, HSP70, in cultured HepG2 cells[8] and rat neuronal cells in vivo.[12] We investigated the effect of exposure to 600 nM TSA or 1 mM valproic acid on GRP78 levels in HEK293 cells by immunoblot analysis (Fig. 2). The results indicate that treatment with 600 nM TSA or 1 mM valproic acid increases GRP78 levels by 2-fold. This is the first indication that TSA can increase GRP78 levels and this result suggests that GRP78 expression is induced by HDAC inhibition.

We investigated the ability of several derivatives of valproic acid to promote histone hyperacetylation in HEK293 cells. Cells were treated with 2 mM 4-phenyl butyric acid, 2-ene-valproic acid or ethyl butyric acid and histone acetylation was determined after 48 h by immunoblot analysis. Results indicate that ethyl butyric acid and 4-phenyl butyric acid do not promote histone hyperacetylation, while 2-ene-valproic acid does promote hyperacetylation (Fig. 3). HDAC inhibition by 2-ene-valproic acid has been previously reported.[20] Next we examined chaperone levels to determine if there is a correlation between HDAC inhibition and chaperone expression (Fig. 4). As a control, cells were treated with 10 μg/ml tunicamycin, an ER stress inducing agent that

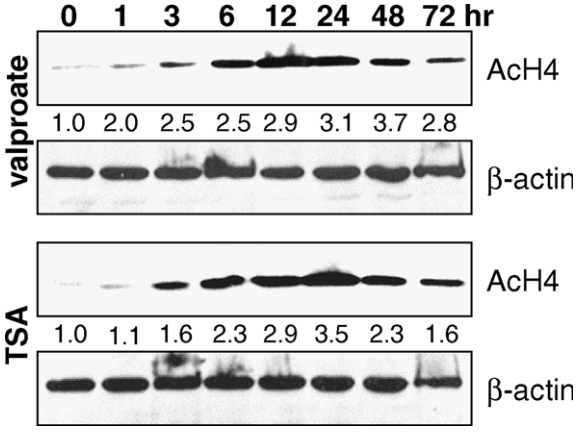


Figure 1. Valproic acid induces hyperacetylation of histone H4. HEK293 cells were cultured in the presence of 1 mM sodium valproic acid or 600 nM TSA for 0–72 h, as indicated. Total protein lysates were resolved by SDS–PAGE, immunostained with antibodies against histone H4 acetylated on lysine 12, and the relative acetylation was quantified by densitometry as indicated. As a loading control, the same blot was immunostained with an antibody against β-actin.

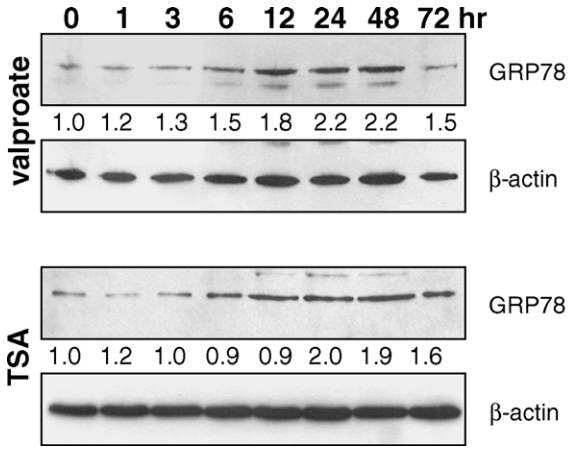
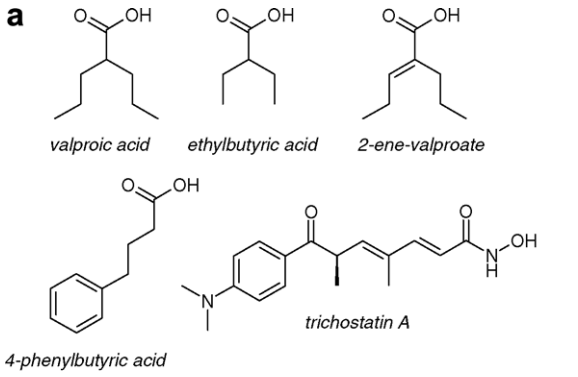


Figure 2. Valproic acid and TSA induce GRP78 protein levels. Total protein lysates, from cells treated with 1 mM valproic acid or 600 nM TSA, were resolved by SDS–PAGE and immunostained with an anti-KDEL antibody that recognizes the ER chaperone GRP78. GRP78 levels were quantified by densitometry. As a loading control, the same blot was immunostained with an antibody against β-actin.



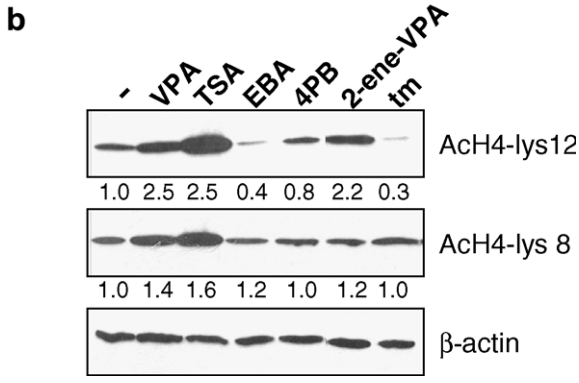


Figure 3. Assaying the ability of valproic acid derivatives to induce histone H4 hyperacetylation. (a) Chemical structures of TSA and derivatives of valproic acid. (b) HEK293 cells were cultured in the presence of valproic acid (VPA, 1 mM), TSA (600 nM), ethyl butyric acid (EBA; 2 mM), 4 phenyl butyric acid (4PB; 2 mM), 2-ene-valproic acid (2-ene-VPA; 2 mM) or tunicamycin (tm; 10 μg/ml) for 48 h. Total protein lysates were probed with antibodies against histone H4 acetylated on lysine 12 or lysine 8 and acetylation was quantified, as indicated.

is known to promote expression of ER chaperones as well as the growth arrest and DNA damage-inducible gene, GADD153/CHOP.[8] Treatment with 1 mM

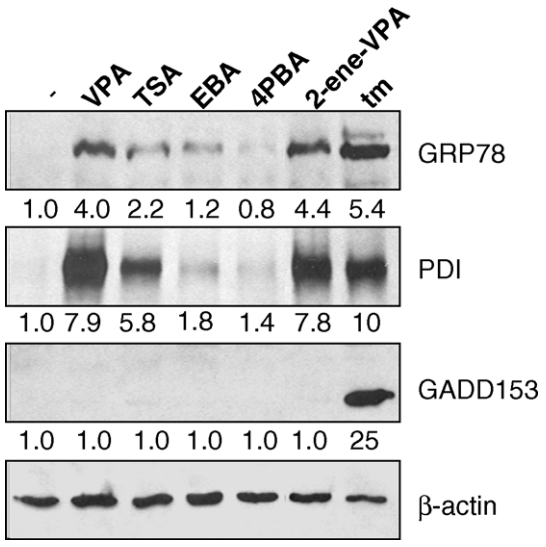


Figure 4. Identifying derivatives of valproic acid that increase chaperone levels and induce ER stress. Total protein lysates from cells treated with valproic acid (VPA), TSA, ethyl butyric acid (EBA), 2-ene-valproic acid (2-ene-VPA), or tunicamycin (tm), as described in Figure 3, were analyzed by immunoblotting with antibodies specific for GRP78, PDI, and GADD153.

valproic acid, 600 nM TSA, 2 mM 2-ene-valproic acid or 10 μg/ml tunicamycin increased the protein levels of GRP78 and PDI. Exposure to 2 mM ethyl butyric acid or 4-phenyl butyric acid had no significant effect on GRP78 or PDI levels. The diagnostic ER stress marker GADD153/CHOP was only induced by tunicamycin suggesting that the other compounds do not promote ER stress and therefore promote chaperone levels by a mechanism that is independent of the ER stress response.

We and others have previously shown that valproic acid can inhibit GSK-3 activity.[8–10,21] To determine if GSK-3 inhibition plays a role in the induction of chaperone levels we measured the effects of ethyl butyric acid, 2-ene-valproic acid, and 4-phenyl butyric acid on GSK-3β activity (Table 1).[22] Consistent with our previous findings, 2 mM ethyl butyric acid and valproic acid significantly inhibit GSK-3β activity. However, 2-ene-valproic acid and 4-phenyl butyric acid did not significantly affect GSK-3β activity. Together these results indicate that the ability to inhibit GSK-3 activity does not correlate with the ability to induce chaperone levels.

ER resident proteins, including GRP78 and PDI, function to assist in the proper folding of nascent proteins in the ER. The folding requirements within the ER are tightly linked to the expression of ER resident chaperones and other factors required for efficient protein processing through the unfolded protein response (UPR). The accumulation of misfolded proteins, a condition known as ER stress, triggers the activation of a transcription factor known as ATF6[23,24] that binds to promoter sequences, called ER stress elements (ERSE), that are found upstream of GRP78 and PDI.[23] Enhanced expression of GRP78, PDI, and other ER stress response proteins increases the folding capacity of the ER.[25,26] The ability of a cell to react to conditions of ER stress through activation of the UPR pathway is essential for the maintenance of ER homeostasis and ultimately, cellular viability.

Table 1. Comparison of GSK-3 inhibition by derivatives of valproic acid

	GSK-3β activity (% of control)
Control	100 ± 9.1
Valproate	65.2 ± 4.4*
Ethyl butyric acid	2.9 ± 0.6*
2-Ene-valproic acid	91.3 ± 9.4
4-Phenyl butyric acid	125.6 ± 35.7

The concentration of each compound = 2 mM. $n \geqslant 3$, $^{*}P < 0.05$.

There is evidence for the existence of mechanisms and pathways that increase ER chaperone expression in the absence of ER stress. For example, the transcription of the genes encoding GRP78 and PDI is induced during early mouse embryonic development by a mechanism that requires the ERSE but appears to be independent of the traditional UPR.[27] Furthermore, in non-stressed myeloid FDC-P1.2 cells, GRP78 and GRP94 expression can be induced by mitogens including interleukin 3 (IL3) and erythropoietin.[28] We and others have shown that the over-expression of GRP78 in the absence of ER stress is cytoprotective. Cells transfected with a transgene encoding GRP78 are resistant to ER stress-induced apoptosis[13,15] and ER stress-induced lipid accumulation.[29]

Gene transcription levels are strongly influenced by the post-translational modification of histones that can directly affect chromatin architecture and DNA packaging. In general, increased acetylation of histones 3 and 4 is associated with increased transcriptional activity, and decreased acetylation is associated with the repression of transcription.[30] Histone acetyltransferases (HAT) and histone deacetylases (HDAC) work in opposition to regulate acetylation levels. The inhibition of HDAC activity results in the hyperacetylation of chromatin and has been associated with the altered transcription/expression of specific genes.[31] It has previously been shown that HDAC inhibitors, including TSA, can promote expression levels of proteins including HSP70,[32] clusterin/apoJ,[33] and calmegin.[34]

Alterations in histone acetylation can have profound effects on cellular metabolism and function. A number of studies have demonstrated that HDAC inhibition can be anti-proliferative and promote the differentiation of cancer cells.[17,31] Aberrant HAT activity has been observed in numerous models of Huntington's disease.[35] Valproic acid is an established HDAC inhibitor[7] and TSA has been shown to effectively compete for binding of valproic acid to HDAC suggesting that it binds to the acetylase active site.[17,36] These observations have increased the interest in the use of valproic acid in the treatment of cancer and other human disease.

Together these findings suggest that valproic acid promotes GRP78, and other protein chaperones, by a

mechanism that involved histone hyperacetylation that is independent of the unfolded protein response. The potential therapeutic benefits of valproic acid-induced chaperone expression in the context of bipolar disorder, epilepsy, and the growing number of human diseases associated with ER stress, including Alzheimer's disease, diabetes mellitus, and atherosclerosis, are yet to be investigated.

Acknowledgments

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18. The human embryonic kidney (HEK) 293 cell line was obtained from the American Type Culture Collection and cultured in DMEM (Life Technologies Inc.) containing 10% fetal bovine serum. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2. Sodium valproate, ethyl butyric acid, trichostatin A (TSA), and tunicamycin were purchased from Sigma Chemical Co. and 4-phenyl butyric acid was purchased from ScienceLabs. The valproic acid derivative, 2-ene-valproic acid, was synthesized as previously described (Ref. 9).
19. Antibodies against PDI (SPA-891) and GRP78 (KDEL, SPA-827) were purchased from StressGen Biotechnologies. Antibodies against acetyl-histone H4 (Lys8) and (Lys12) were purchased from Upstate. The antibody against GADD153/CHOP was purchased from Santa Cruz Biotechnology and the anti-β-actin antibody was obtained from Sigma Chemical Co. Total protein lysates from cultured cells were solubilized in SDS–PAGE sample buffer and equivalent amounts of total protein were separated on SDS–polyacrylamide gels under reducing conditions, as described previously.[8] After incubation with the appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Life Technologies Inc.), the membranes were developed using ECL Plus (Amersham) and quantified using a Typhoon 9410 Imaging System (Amersham) and normalized to β-actin. Results are presented as means of at least three independent experiments. Representative immunoblots are shown.
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