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Valproate Induces the Unfolded Protein Response by Increasing Ceramide Levels*[S]

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Bipolar disorder (BD), which is characterized by depression and mania, affects 1–2% of the world population. Current treatments are effective in only 40–60% of cases and cause severe side effects. Valproate (VPA) is one of the most widely used drugs for the treatment of BD, but the therapeutic mechanism of action of this drug is not understood. This knowledge gap has hampered the development of effective treatments. To identify candidate pathways affected by VPA, we performed a genome-wide expression analysis in yeast cells grown in the presence or absence of the drug. VPA caused up-regulation of *FEN1* and *SUR4*, encoding fatty acid elongases that catalyze the synthesis of very long chain fatty acids (C24 to C26) required for ceramide synthesis. Interestingly, *fen1*Δ and *sur4*Δ mutants exhibited VPA sensitivity. In agreement with increased fatty acid elongase gene expression, VPA increased levels of phytoceramide, especially those containing C24–C26 fatty acids. Consistent with an increase in ceramide, VPA decreased the expression of amino acid transporters, increased the expression of ER chaperones, and activated the unfolded protein response element (*UPRE*), suggesting that VPA induces the UPR pathway. These effects were rescued by supplementation of inositol and similarly observed in inositol-starved *ino1*Δ cells. Starvation of *ino1*Δ cells increased expression of *FEN1* and *SUR4*, increased ceramide levels, decreased expression of nutrient transporters, and induced the UPR. These findings suggest that VPA-mediated inositol depletion induces the UPR by increasing the *de novo* synthesis of ceramide.

Bipolar disorder (BD),[2] one of the most severe forms of mood disorder, is characterized by recurrent episodes of depression and mania (1, 2). BD is ranked as the sixth leading cause of disability worldwide. It affects about 1–2% of the total world population (1–3) and leads to suicide in 15% of cases (4). Valproic acid (VPA), a branched short-chain fatty acid, is one of the most widely used drugs for the treatment of BD. However, it is effective in only 40–60% of cases and results in serious side effects, including hepatotoxicity and teratogenicity (5). Although many hypotheses have been postulated to explain its efficacy, the therapeutic mechanism of the drug is not understood, nor is the underlying cause of the disease (6, 7). This knowledge gap hampers the development of more effective drugs to treat BD.

The inositol depletion hypothesis has had a major impact on research in BD. Berridge (8) proposed that lithium, widely used to treat BD, inhibits inositol monophosphatase, causing inositol depletion and subsequently decreasing inositol 1,4,5-triphosphate-mediated signaling (8, 9). We have shown previously that VPA, similar to lithium, causes a decrease in intracellular inositol in yeast and mammalian cells (10–12). VPA indirectly inhibits *myo*-inositol-3-phosphate synthase (MIPS), the enzyme responsible for the rate-limiting step of *de novo* synthesis of inositol, suggesting that MIPS is post-translationally regulated (13). More recent findings show that yeast and human MIPS are phosphorylated (14, 15) and that phosphorylation of conserved sites affects enzymatic activity (14). These findings suggest that the mechanism by which VPA causes inositol depletion is conserved in yeast and mammals, supporting the yeast model for genetic and molecular studies of the mechanism of the drug.

In yeast, supplementation of inositol triggers a change in the expression of hundreds of inositol-regulated genes, including genes for lipid synthesis (16–18). Inositol-containing lipids, including inositol phosphoinositides, glycosylphosphatidylinositol, and sphingolipids, play crucial structural and functional roles in regulating membrane biogenesis, membrane trafficking, cytoskeletal organization, and gene expression (19–23). Hence, inositol depletion exerts profound effects on cellular function (24). Inositol depletion not only alters lipid biosynthesis (25) but also activates stress response pathways, including the protein kinase C and unfolded protein response (UPR) pathways (26–28). Cells grown in the absence of inositol exhibit induction of the UPR pathway (27, 29), which is reversed by inositol supplementation (16). These studies suggest that decreasing the intracellular levels of inositol induces the UPR pathway by a mechanism not yet characterized.

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[S] This article contains supplemental Table 1.

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[2] The abbreviations used are: BD, bipolar disorder; VPA, valproic acid; ER, endoplasmic reticulum; UPR, unfolded protein response; PHC, phytoceramide; DHC, dihydroceramide; LCB, long chain base; MIPS, *myo*-inositol-3-phosphate synthase; qRT-PCR, quantitative RT-PCR.

Interdependence of the UPR pathway and ceramide synthesis has been demonstrated in yeast and mammals. Induction of the UPR increases ceramide levels and viability in yeast (30). In mammals, activation of the UPR increases the expression of ceramide synthase CerS6, leading to increased synthesis of ceramides containing C16 fatty acids (30). Perturbation of *de novo* synthesis of sphingolipids activates the UPR in yeast (29, 31), and defective ceramide homeostasis leads to UPR failure (32). Ceramide also decreases the transcription of nutrient transporters, including amino acid transporter *mCAT-1*, glucose transporter *GLUT-1* (33), glucose transporter *HXT4*, and uracil permease *FUR4* (34). Therefore, the interrelationship between ceramide levels and the UPR pathway maintains cell homeostasis.

In the current study, we show for the first time that VPA-mediated inositol depletion induces the UPR pathway by increasing *de novo* synthesis of ceramide, especially C24–C26-containing phytoceramide. These findings have implications for the therapeutic mechanism of VPA.

Results

VPA Increases the Expression of Fatty Acid Elongases—To determine candidate pathways that may be important for the therapeutic role of VPA, we performed a genome-wide microarray analysis of cells treated with 0.6 mM VPA for 5 h in the presence or absence of inositol, as described under "Experimental Procedures." VPA treatment resulted in altered expression (>2-fold) of 324 genes in the presence of inositol and 413 genes in the absence of inositol (supplemental Table 1). Interestingly, fatty acid elongase genes *FEN1* and *SUR4* exhibited 2-fold increased expression in response to VPA (supplemental Table 1). qRT-PCR analysis of fatty acid elongase genes in WT cells treated with VPA validated these findings. As seen in Fig. 1, mRNA levels of *FEN1* and *SUR4* were increased 6- and 4-fold in the absence of inositol and to a lesser extent (3- and 2-fold) in the presence of inositol. Fen1 and Sur4 catalyze the synthesis of very long chain fatty acids, including C22 and C24 (Fen1) and C24 and C26 (Sur4) (35), which are used for the synthesis of ceramide. However, VPA did not alter the expression levels of *ELO1*, a fatty acid elongase that catalyzes the synthesis of C16 or C18 (Fig. 1). This indicates that VPA specifically increases the expression of fatty acid elongases catalyzing the synthesis of very long chain fatty acids (C24–C26). Mutants *fen1*Δ and *sur4*Δ exhibited sensitivity to VPA, as did the sphinganine C4-hydroxylase mutant, *sur2*Δ (Fig. 2). VPA sensitivity was partially rescued by inositol. The VPA-mediated increase in the expression of these genes and the VPA sensitivity of Fen1, Sur4, and Sur2 mutants suggested that VPA induces an increase in ceramide containing C24–C26 fatty acids and phytosphingosine (the product of Sur2).

VPA Increases Ceramide Levels and Down-regulates Amino Acid Transporters—To determine whether VPA increases ceramide levels, dihydroceramide (DHC) and phytoceramide (PHC) ceramide species were analyzed by mass spectrometry in WT cells. VPA showed a trend of increase in ceramide levels, especially C26 phytoceramide, but this increase was not statistically significant (Fig. 3*B*). Interestingly, VPA treatment for 30 min up-regulated the expression of *RSB1* (data not shown), which

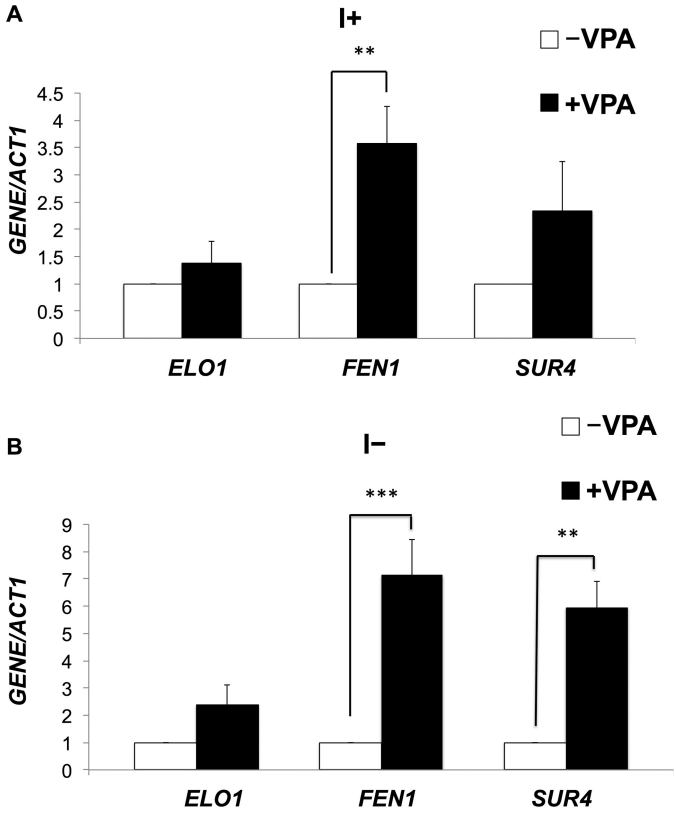

FIGURE 1. **VPA up-regulates expression of fatty acid elongase genes.** WT cells were precultured in I+ medium, harvested, washed twice with sterile water, and grown in I+ or I− medium until cells reached the mid-log phase (A_{550} = 0.5). Cells were pelleted and suspended in fresh I− medium with or without 0.6 mM VPA and incubated for 5 h. mRNA levels of fatty acid elongases were quantified by qRT-PCR in WT cells grown in the presence or absence of VPA. Values are reported as -fold change in expression in cells grown in VPA relative to cells grown without VPA, in the absence (*A*) or presence of inositol (*B*). Expression was normalized to the mRNA levels of the internal control *ACT1*. Data shown are mean ± S.D. (*error bars*) (n = 6) (**, $p < 0.01$; ***, $p < 0.001$).

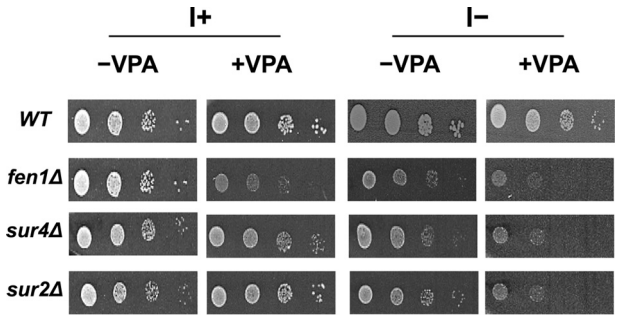

FIGURE 2. **VPA sensitivity of fatty acid elongase and sphinganine C4-hydroxylase mutants.** Cells were precultured in I+, counted using a hemocytometer, and washed with sterile water. 3-μl aliquots of a series of 10-fold dilutions were spotted onto I+ or I− plates in the presence or absence of 1 mM VPA and incubated for 3 days at 30 °C.

transports long chain bases (LCBs) (including dihydrosphingosine and phytosphingosine) across the plasma membrane from the inside to the outside of the cell (36). In WT cells, up-regulation of *RSB1* increases the transport of LCBs, reducing the intracellular levels of these ceramide precursors. Therefore, we measured the effect of VPA on ceramide levels with varying fatty acids in *rsb1*Δ cells. VPA increased both DHC and PHC in *rsb1*Δ (Fig. 3). Specific ceramide species were increased, including DHC with C16, C18,

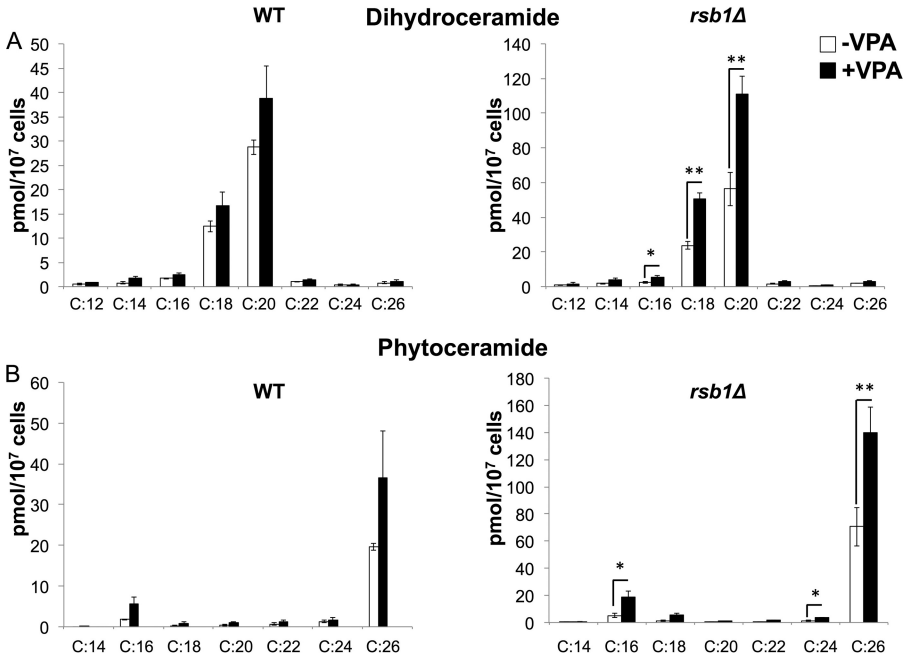

FIGURE 3. **VPA increases levels of DHC and PHC.** WT and *rsb1*Δ cells were grown as described in the legend to Fig. 1. Cells were pelleted, and DHC (*A*) and PHC (*B*) levels with varying fatty acids were quantified by mass spectrometry. Data shown are mean ± S.D. (*error bars*) ($n = 6$; *, $p < 0.05$; **, $p < 0.01$).

and C20 and PHC with C16, C24, and C26 in *rsb1*Δ cells (Fig. 3). The highest increase was observed in C26 species of PHC. The likely explanation for this is that the increase in *RSB1* in WT cells reduces the available LCB precursor for ceramide synthesis. However, when *RSB1* is deleted, the increase in ceramide levels is more significant.

Previous studies have shown that increased levels of ceramide cause a decrease in the transcription of nutrient permeases, leading to reduced intake of nutrients and induction of stress (33, 34). Consistent with this, the microarray analysis revealed decreased expression of amino acid transporter genes *BIO5*, *AGP1*, *GAP1*, *BAP2*, *DIP5*, *UGA4*, *CAN1*, and *SAM3* in response to VPA in the absence of inositol. The expression of these transporters was not altered in I+ medium (supplemental Table 1). Decreased expression of amino acid transporters was confirmed by qRT-pCR in both WT and *rsb1*Δ cells (Fig. 4). Down-regulation was greater in *rsb1*Δ than in WT cells, consistent with the increase in ceramide levels. To determine whether decreased expression of amino acid transporters resulted from increased ceramide, cells were treated with 100 μM fumonisin, a ceramide synthase inhibitor (37, 38). Fumonisin reversed the down-regulation of the amino acid transporters in both WT and *rsb1*Δ cells (Fig. 4). Taken together, these findings suggest that VPA increases levels of specific ceramide species, resulting in decreased expression of nutrient permeases.

VPA Induces the UPR by Increasing Ceramide Levels—Decreased expression of nutrient transporters is expected to induce stress due to nutrient starvation (33). In agreement with this, VPA increased the expression of ER chaperone genes *EUG1*, *JEM1*, *KAR2*, *LHS1*, *SEC63*, and *PDI1* in I− medium (supplemental Table 1 and Fig. 5), suggesting that the UPR pathway was induced. To test this, we analyzed the UPR response in WT and *rsb1*Δ cells expressing a *UPRE-lacZ* reporter plasmid. Increased *lacZ* activity was observed in response to VPA in I− medium (Fig. 6*B*). The increase was more pronounced in *rsb1*Δ than in WT cells. Fumonisin, a

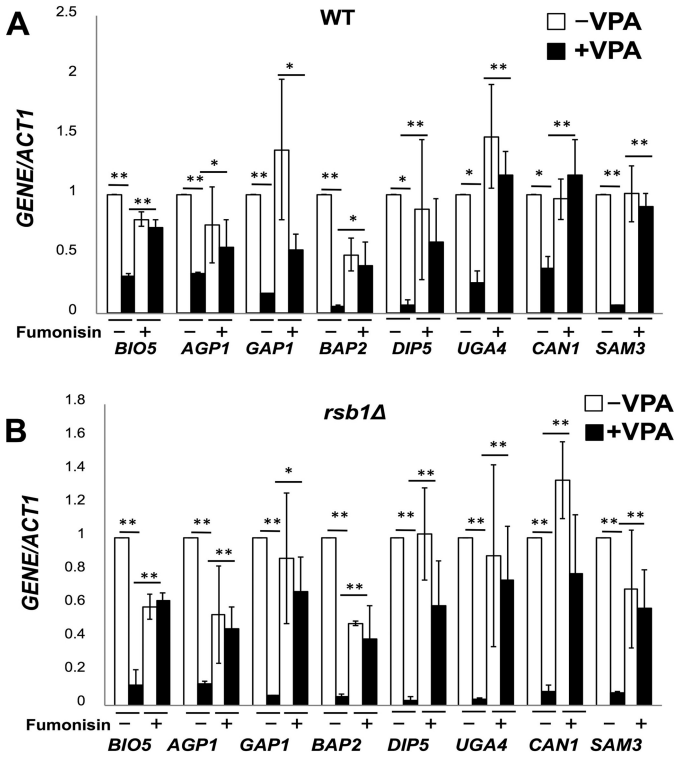

FIGURE 4. **VPA down-regulates expression of nutrient transporters via ceramide.** mRNA levels of nutrient transporters were quantified by qRT-PCR in WT (*A*) and *rsb1*Δ (*B*) cells grown in the presence or absence of VPA and fumonisin, as indicated. Values are reported as -fold change in expression in cells grown in VPA relative to cells grown without VPA. Expression was normalized to the mRNA levels of the internal control *ACT1*. Data shown are mean ± S.D. (*error bars*) ($n = 6$) (*, $p < 0.05$; **, $p < 0.01$).

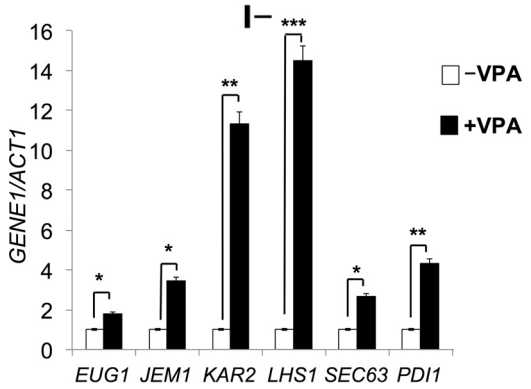

FIGURE 5. **VPA increases expression of ER chaperones.** WT cells were grown as described in the legend to Fig. 1. mRNA levels of chaperone genes were quantified by qRT-PCR in wild type cells grown in I− medium in the presence or absence of VPA. Values are reported as -fold change in expression in cells grown in VPA relative to cells grown without VPA. Expression was normalized to the mRNA levels of the internal control *ACT1*. Data shown are mean ± S.D. (*error bars*) ($n = 6$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ceramide synthase inhibitor (Fig. 6, *A* and *B*) restored *lacZ* activity to normal levels. In contrast, aureobasidin, which inhibits the conversion of ceramide to complex sphingolipids (Fig. 6*A*), did not affect *UPRE* expression (Fig. 6*C*). Increased *UPRE* expression was not observed in I+ medium (Fig. 6*D*). These findings suggest that VPA induced the UPR pathway via increased *de novo* synthesis of ceramide and not by inhibiting the conversion of ceramide to complex sphingolipids.

Consistent with previous reports showing that inositol starvation induces the UPR pathway (27, 29), *UPRE* expression was increased in inositol-starved *ino1*Δ mutant cells (Fig. 7*A*). Treatment with fumonisin decreased *UPRE* expression to levels observed in control cells, indicating that inositol starvation of *ino1*Δ cells induces the UPR pathway by increasing ceramide levels.

Similar to VPA treatment, *ino1*Δ cells starved for inositol for 3 h also exhibited decreased expression of amino acid transporter genes *BIO5*, *AGP1*, *GAP1*, *BAP2*, *DIP5*, *UGA4*, *CAN1*, and *SAM3* (Fig. 7*B*). This decrease was ceramide-dependent because it was blocked by fumonisin. Decreased expression of amino acid transporter genes *BAP2*, *GAP1*, and *UGA4* was partially rescued by fumonisin (Fig. 7*B*), suggesting that inositol depletion decreased the expression of amino acid transporter genes, at least in part, by increasing ceramide levels.

To directly test the hypothesis that inositol depletion decreased the expression of amino acid transporters and induced the UPR by increasing the intracellular ceramide levels, *ino1*Δ cells were starved for 3 h, and ceramide levels were quantified (Fig. 7*C*). Inositol starvation increased levels of some DHC and PHC species (Fig. 7*C*). Most notable were increases in DHC with C18 and C20 and PHC with C26 (Fig. 7*C*). The highest increase was observed in C26 species of PHC, similar to the response to VPA (Fig. 3). Expression of fatty acid elongases *FEN1* and *SUR4* was also increased during inositol starvation (Fig. 7*D*).

Taken together, our findings indicate that inositol depletion mediated by VPA or by starvation of *ino1*Δ cells led to increased expression of fatty acid elongases (elongating C24–C26), resulting in increasing ceramide levels with C24–C26 fatty acids. Increased ceramide synthesis led to down-regulation of transporters and induction of the UPR pathway. These findings are the first to show that VPA-mediated inositol depletion induces the UPR by increasing ceramide levels.

Discussion

In this study, we show that VPA induces the UPR by increasing ceramide levels via inositol depletion. Our specific findings indicated that VPA 1) increased expression of fatty acid elongases synthesizing C24 and C26 fatty acids; 2) increased ceramide levels, particularly PHC with C24–C26 fatty acids; 3) decreased the expression of amino acid transporters; and 4) induced the UPR pathway. These outcomes were abrogated by inhibition of *de novo* ceramide synthesis or by supplementation of inositol.

Ceramide levels can be increased via two routes, increased *de novo* synthesis by ceramide synthase or inhibition of inositol phosphorylceramide synthase, which converts ceramide to complex sphingolipids. Our findings suggest that VPA induces the UPR pathway by increasing *de novo* synthesis of ceramide (Fig. 6*B*) as a result of increased expression of fatty acid elongases, because induction did not occur in the presence of the ceramide synthase inhibitor fumonisin (37). In contrast, aureobasidin, which inhibits inositol phosphorylceramide synthase, did not affect VPA-mediated induction of the UPR (Fig. 6*C*).

Our findings are consistent with studies showing that inositol starvation induces the UPR. UPR target genes were found to be significantly up-regulated in cells grown in the absence of inositol (16). In addition, inositol supplementation alters the expression of UPR pathway genes (39). Although initial studies showed that accumulation of unfolded proteins in the ER induces the UPR pathway (40, 41), it has since been determined that Ire1p, the transmembrane kinase that senses ER stress (27, 42), induces the UPR pathway in response to changes in membrane lipid composition (29). In this light, inositol starvation may trigger the UPR as a result of lipid-related membrane changes in the ER, including perturbation of sphingolipid metabolism. The addition of inositol induces changes in the synthesis and levels of numerous lipids, including sphingolipids (28, 43). Recent studies show that perturbation of sphingolipid metabolism may affect ER membrane homeostasis. Mutant cells lacking *ORM1* and *ORM2*, negative regulators of sphingolipid metabolism (31, 44), exhibit constitutive induction of the UPR response (31). In addition, *orm1*Δ *orm2*Δ cells contain elevated levels of sphingolipids (31, 44) and are hypersensitive to stress induced by inositol starvation (31). Similarly, UPR expression is constitutive in *isc1*Δ mutant cells, which contain elevated sphingolipids due to a block in sphingolipid turnover (45, 46). Conversely, inhibiting sphingolipid synthesis with myriocin treatment suppresses activation of the UPR induced by inositol starvation (29). Elevated sphingolipids in the ER may lead to membrane aberrancy that activates the UPR pathway independent of accumulation of unfolded protein. The role of specific sphingolipids in activation of the UPR is not known.

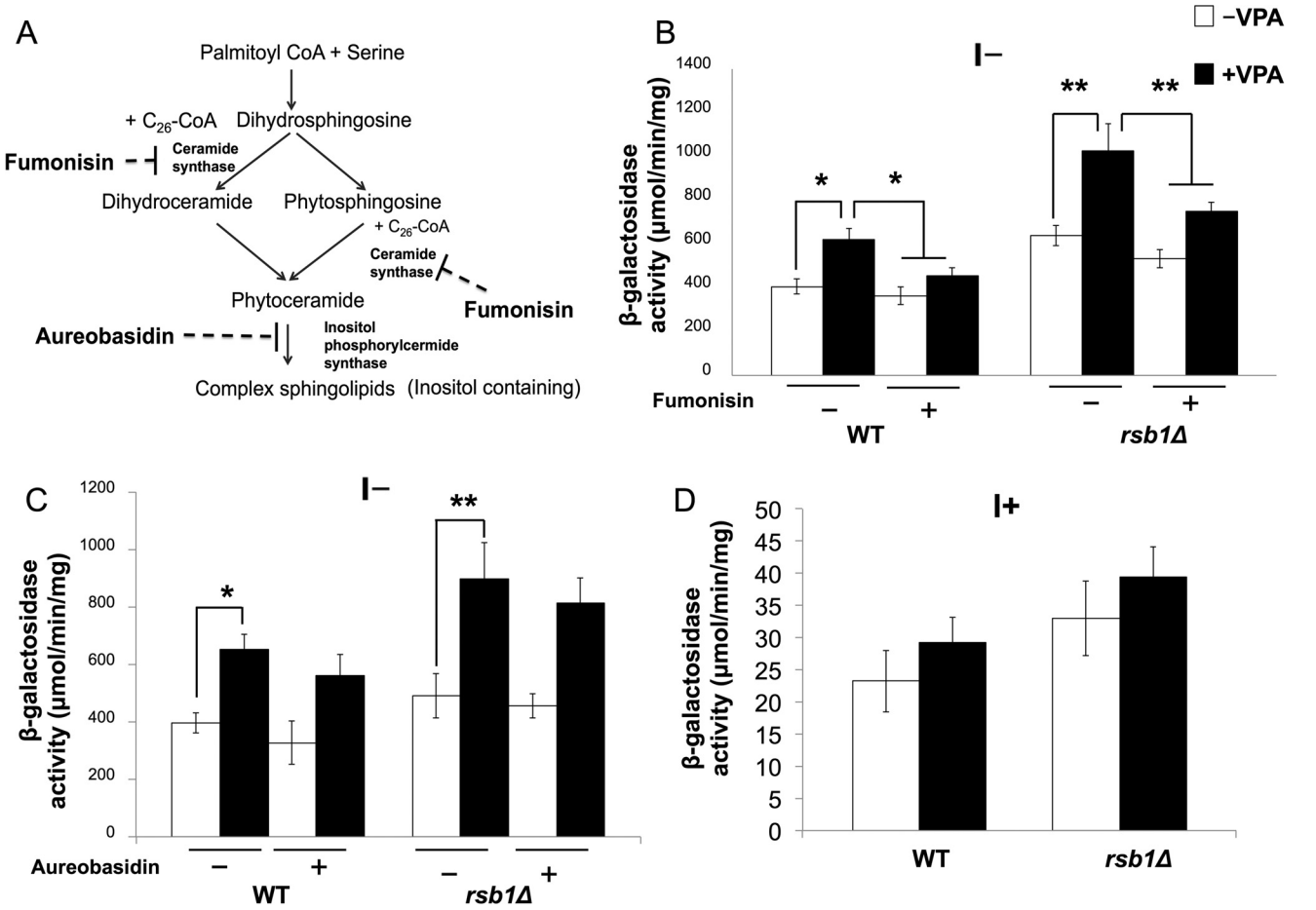

FIGURE 6. **VPA up-regulates *UPRE* expression.** *A*, schematic of sphingolipid synthesis. Serine is condensed with palmitoyl-CoA to form 3-ketodihydrosphingosine, which is reduced to dihydrosphigosine. Dihydrosphingosine is hydroxylated on C4 to produce phytosphingosine. Ceramide synthase catalyzes the linkage of very long chain fatty acids produced by fatty acid elongases to dihydrosphingosine and phytosphingosine to form ceramide (dihydroceramide or phytoceramide). Ceramide is used as a precursor for the synthesis of inositol containing complex sphingolipids. Inositol phosphorylceramide synthase catalyzes the first step of complex sphingolipid synthesis. Fumonisin is a ceramide synthase inhibitor, and aureobasidin inhibits inositol phosphorylcermide synthase. *B*, WT and *rsb1*Δ cells expressing a *UPRE-lacZ* plasmid were precultured in Ura-I+ medium and then grown in Ura-I− medium with or without 100 μM fumonisin or 0.5 μg/μl aureobasidin (*B* and *C*) or Ura-I+ medium (*D*). 0.6 mM VPA was added when indicated. *LacZ* activity is quantified as indicated. Data shown are mean ± S.D. (*error bars*) ($n = 6$) (*, $p < 0.05$; **, $p < 0.01$).

Our study suggests that accumulation of ceramide, specifically C24–C26-containing PHC, induces the UPR upon inositol depletion.

We have previously shown that VPA decreases intracellular inositol levels (10–13, 47). VPA induction of the UPR occurred only in I− conditions, suggesting that the response to VPA was mediated by inositol depletion. Consistent with this, inositol starvation of *ino1*Δ cells caused similar effects, including increased expression of fatty acid elongases, increased intracellular levels of ceramides, especially PHC with C24 and C26, down-regulation of nutrient transporters, and induction of the UPR. Based on these data, we propose the following model (Fig. 8). VPA decreases intracellular inositol levels, causing an increase in C24 and C26 PHC species as a result of up-regulation of expression of fatty acid elongases (*FEN1* and *SUR4*). This results in increased ceramide synthesis, which causes down-regulation of expression of nutrient transporters, inducing stress and the UPR.

Several studies suggest that the UPR pathway may be involved in the pathophysiology of BD. Lymphoblasts from bipolar patients show an aberrant ER stress response (48, 49). Compared with cells from healthy controls, lymphoblasts from bipolar patients had lower expression of *XBP1* (mammalian homologue of yeast *HAC1*), the spliced form of which binds the *UPRE* element and induces the UPR pathway in response to ER stress inducers (49). A polymorphism (−116C → G) in the XBP1 promoter that is associated with lower gene transcription was observed in BD lymphoblastoid cells (50). These findings suggest that the UPR pathway may be perturbed in bipolar patients, and activation of this pathway may be important for the therapeutic action of VPA. In this light, the UPR pathway may be a possible new target for BD therapy.

Experimental Procedures

Yeast Strains, Growth Media, and Conditions—Strains used in this study are summarized in Table 1. Cells were maintained on YPD medium (2% glucose, 1% yeast extract, 2% bactopeptone). Deletion mutants were maintained on medium supplemented with G418 (200 μg/ml). Synthetic minimal medium without inositol (I−) contained all of the essential components of Difco yeast nitrogen base (minus

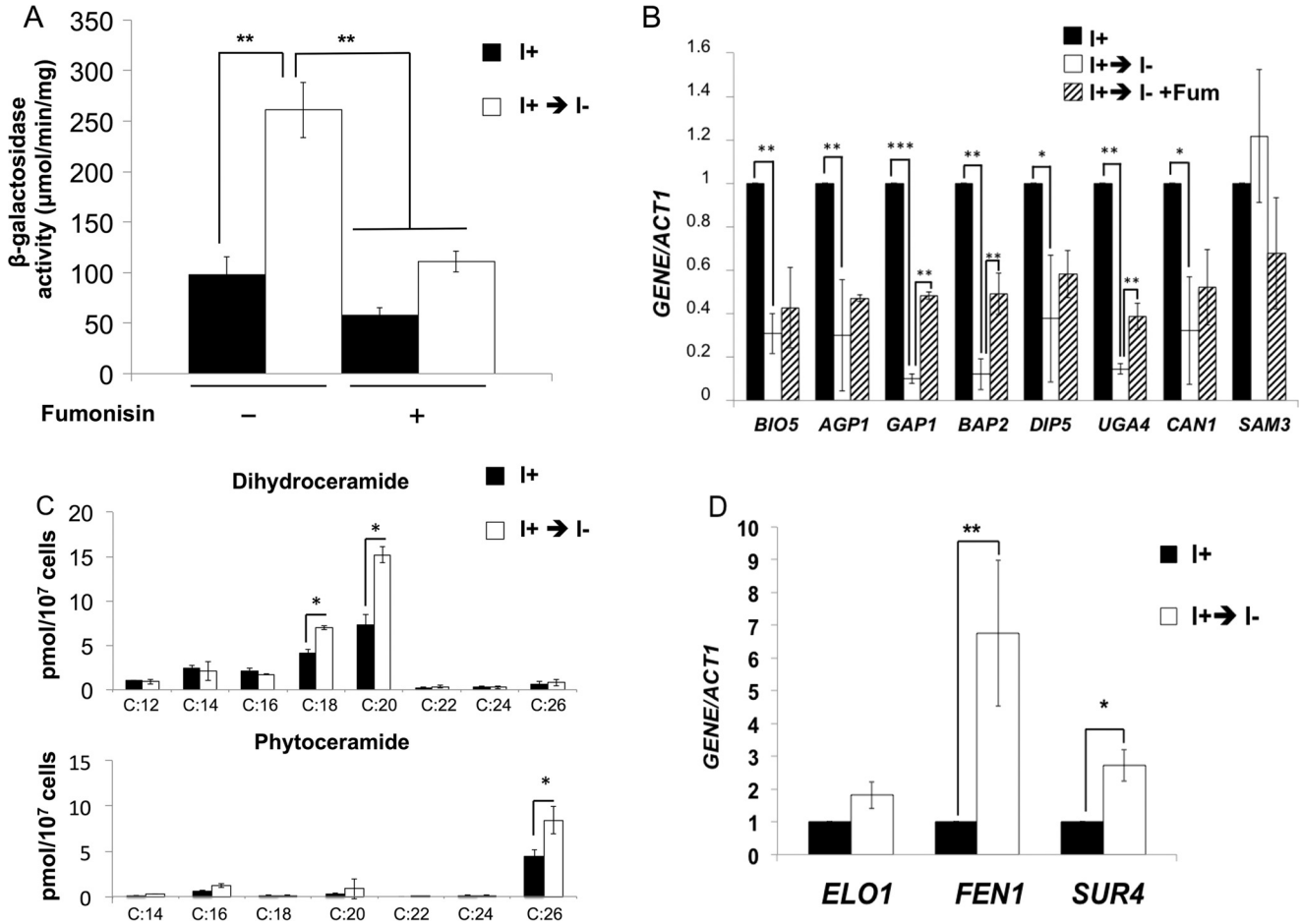

FIGURE 7. **VPA induces the UPR by inositol depletion.** *ino1*Δ cells expressing the *UPRE-lacZ* reporter plasmid were precultured and grown in Ura-I+ medium until the cells reached mid-log phase (A_{550} = 0.5). Cells were washed and transferred to Ura-I− medium with or without 100 μM fumonisin for 3 h. *A*, *LacZ* activity. Data shown are mean ± S.D. (*error bars*) (n = 6) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *B*, DHC and PHC levels with varying fatty acids were quantified by mass spectrometry. Data shown are mean ± S.D. (n = 6) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). mRNA levels of nutrient transporters (*C*) and fatty acid elongases (*D*) were quantified by qRT-PCR, as indicated. Values are reported as -fold change in expression in cells grown in I− medium relative to cells grown in I+ medium. Expression was normalized to the mRNA levels of the internal control *ACT1*. Data shown are mean ± S.D. (n = 6). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

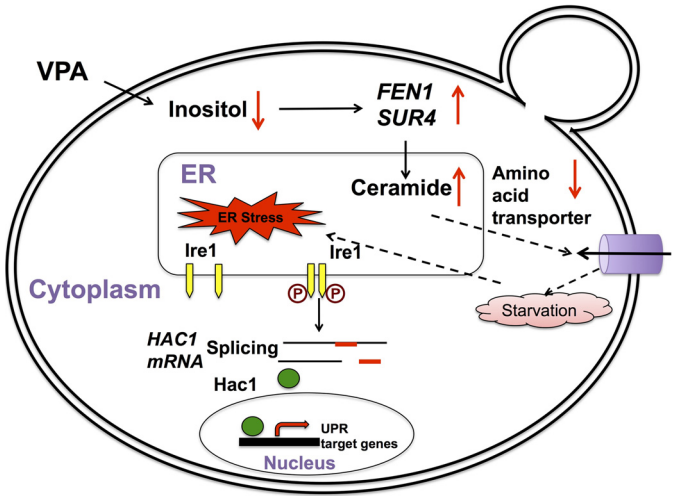

FIGURE 8. **Model; VPA induces the UPR pathway by increasing intracellular ceramide levels.** In the proposed model, VPA-mediated inositol depletion leads to increased expression of fatty acid elongases, specifically *FEN1* and *SUR4*, which catalyze the synthesis of C24 and C26 fatty acids and ceramides, especially PHC containing C24–C26 fatty acids. Increased ceramide levels decrease expression of nutrient transporters, stressing the cell due to lack of nutrients and inducing the UPR pathway.

inositol); 2% glucose; 0.2% ammonium sulfate; vitamins; the four amino acids histidine (20 mg/liter), methionine (20 mg/liter), leucine (60 mg/liter), and lysine (20 mg/liter); and the nucleobase uracil (40 mg/liter). Where indicated, inositol (I) was added at a concentration of 75 μM. For selection of plasmids, uracil was omitted. Liquid and solid media were supplemented with 0.6 and 1 mM VPA, respectively, when indicated. Fumonisin B1 (Sigma) and aureobasidin A (Clontech) were used at a concentration of 100 μM and 0.5 μg/μl, respectively. For solid media, 2% agar was added. Absorbance was measured at 550 nm to monitor growth in liquid cultures. All incubations were at 30 °C.

VPA Treatment—WT cells were precultured in synthetic minimal medium with inositol (I+), harvested, washed twice with sterile water, and grown in I+ until the cells reached the mid-log phase (A_{550} = 0.5). Cells were pelleted, washed twice with sterile water and inoculated in I+ or I− to a final A_{550} of 0.05 and cultured until the cells reached the mid-log phase (A_{550} = 0.5). Cells were then pelleted and suspended in fresh I− or I+ medium with or without 0.6 mM VPA and incubated for 5 h.

TABLE 1
Yeast strains and plasmids used in this study

Strain/Plasmid	Genotype/Description	Source/Reference
Wild type	MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura3Δ0	Invitrogen
*fen1*Δ	MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, fen1Δ::KanMX4	Invitrogen
*sur4*Δ	MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, sur4Δ::KanMX4	Invitrogen
*rsb1*Δ	MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, rsb1Δ::KanMX4	Invitrogen
*ino1*Δ	MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, ino1Δ::KanMX4	Invitrogen
RSB1-HA	pRS316-RSB1Δ335–382–3 × HA	Johnson *et al.* (55)
UPRE-LacZ	pJC104, containing UPRE-CYC-lacZ	Chang *et al.* (53)

TABLE 2
Real-time PCR primers used in this study

Gene	Primer	Sequence (5′–3′)
ACT1	Forward	ACGTTCCAGCCTTCTACGTTTCCA
ACT1	Reverse	ACGTGAGTAACACCATCACCGGAA
FEN1	Forward	TGGGTTCAACAACTGCCACCTTTG
FEN1	Reverse	TCATTAACCTTTGCGGCAACACCG
SUR4	Forward	TGTTATGGTACTCAGGCTGCTGCT
SUR4	Reverse	AGTAGAAGAACCGGATGCAACGGA
RSB1	Forward	TTGCCCTCTCCAATGGCGTATTCT
RSB1	Reverse	ACATGATTGCCGGTTGTTGTGGAC
ELO1	Forward	AGAAAGCCTCTAGGTTTCGCCCAA
ELO1	Reverse	AAAGGCTGCTTCCCAACGGTAAAC
BIO5	Forward	GCATCCGGACTACGAGTTAAAG
BIO5	Reverse	GGGCAACGGAGTTGAATAAATG
AGP1	Forward	GAACGATCTTACGTCGGCTATC
AGP1	Reverse	GACCTGTATTAGCGCCTATGTT
GAP1	Forward	GTGACACTCCAGGTGCTAAA
GAP1	Reverse	GCAGCAAGACCAACCAATTC
BAP2	Forward	GAGGATGGCGTTGAGTCTATC
BAP2	Reverse	GTCCCAATACCTGTCCCTAAAG
DIP5	Forward	TCATTTCTTGGGCTGGTTACA
DIP5	Reverse	GGTCCTTCATTCTTCCCTCTTC
UGA4	Forward	TGGTGGTCCAGCAACATTAG
UGA4	Reverse	AGCGGTAGGAATGGAACTTG
CAN1	Forward	GAACGCTGAAGTGAAGAGAGAG
CAN1	Reverse	GTTGGTCAGAGGTGTGGATAAA
SAM3	Forward	GATGTATCTGCCTCTCCCTTTG
SAM3	Reverse	CACAACCGCAACAAGGATAAC
EUG1	Forward	TGGTCAAGTCTATCGCGGTGTCAA
EUG1	Reverse	CATTCAAGCCTGTCAAGCCTCTGT
JEM1	Forward	TGGGACAAGGTGCATCAGAAGGAT
JEM1	Reverse	GCGTTATGCGTAGCAGCTCAGAAA
KAR2	Forward	AAAGATGGGAAGCCCGCTGTAGAA
KAR2	Reverse	ACAGCATGGGTAACCTTAGTGCCT
LHS1	Forward	GCGCGGAAGTGCTTATCCAAACAA
LHS1	Reverse	ACGCAACTCCTGACGAGCACTTAT
SEC63	Forward	AGCAAAGGGCCTAACACCTGATGA
SEC63	Reverse	TGGGCCATCTGGATGACCGTATTT
PDI1	Forward	TGCCATCCACGACATGACTGAAGA
PDI1	Reverse	ACTCCAACACGATCTTGTCGCTCA

*ino1*Δ *Starvation*—*ino1*Δ cells were precultured in I+, harvested, washed twice with sterile water, and grown in I+ until the cells reached mid-log phase (A_{550} = 0.5). Cells were pelleted, washed twice with sterile water, and transferred to fresh I− (inositol starvation) or I+ (control) for 3 h.

Microarray Analysis—Total RNA was isolated by hot phenol extraction (51) and purified using an RNeasy kit from Qiagen. Quality of RNA was determined using Agilent 2100 Bioanalyzer. RNA was labeled using the Agilent Low Input QuickAmp labeling kit (Agilent Technologies). Cy3-labeled cRNA was then hybridized to the 8 × 15K Agilent Yeast V2 Arrays (design ID 016322). Slides were scanned on an Agilent G2505B microarray scanner, and the resulting image files were processed with Agilent Feature Extraction software (version 9.5.1). All procedures were carried out according to the manufacturer's protocols. Subsequent analysis was performed using GeneSpring (version 10.0) software. Microarray analysis was carried out at the Research Technology Support Facility at Michigan State University.

qRT-PCR Analysis—Total RNA was extracted using the hot phenol method (51) and purified using an RNeasy mini plus kit (Qiagen, Valencia, CA). cDNA was synthesized using the first strand cDNA synthesis kit from Roche Applied Science as described in the manufacturer's manuals. qRT-PCRs were done in a 20-μl volume reaction using Brilliant III Ultra-Faster SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA). Each reaction was done in triplicate. The primers used for the qRT-PCRs are listed in Table 2. RNA levels were normalized to *ACT1* levels (internal control). Relative values of mRNA transcripts are shown as -fold change relative to the indicated controls. Primers were validated as suggested in the Methods and Applications Guide (Agilent Technologies). All primers used in this study had primer efficiency between 85 and 105%. Optimal primer concentrations were determined, and primer specificity of a single product was monitored by a melt curve following the amplification reaction. PCRs were initiated at 95 °C for 10 min for denaturation followed by 40 cycles consisting of 30 s at 95 °C and 60 s at 55 °C.

Ceramide Measurement—Cells were grown and treated with VPA as described above for 5 h, pelleted, and stored at −80 °C. Extraction of lipids from yeast pellets and lipid quantification by LC/MS/MS was performed as described previously (52).

β-Galactosidase Assays—Cells expressing the *UPRE-LacZ* reporter plasmid provided by Dr. Susan Henry (53) were precultured in Ura-I+ and grown in Ura-I+ to an A_{550} of 0.5, washed, and transferred to Ura-I− medium with or without VPA for 5 h at 30 °C. Cells were harvested, and β-galactosidase was assayed as described (54).

Author Contributions—S. J. and M. L. G. designed the research and wrote the manuscript; S. J. carried out all of the experiments; S. R., S. C., R. S., and A. C. performed ceramide measurements; S. J., A. C., R. S., and M. L. G. carried out the data analysis. All authors reviewed the results and approved the final version of the manuscript.

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