Valproic Acid Is a Novel Activator of AMP-Activated Protein Kinase and Decreases Liver Mass, Hepatic Fat Accumulation, and Serum Glucose in Obese Mice

Lindsay B. Avery and Namandjé N. Bumpus

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Received September 18, 2013; accepted October 8, 2013

ABSTRACT

Valproic acid (VPA) is a widely prescribed anticonvulsant for the treatment of epilepsy. Here we demonstrate that VPA is a novel activator of AMP-activated protein kinase (AMPK), a key regulator of cellular metabolism, using primary mouse and human hepatocytes. Incubation of primary mouse hepatocytes with VPA resulted in increased levels of phosphorylated AMPK and acetyl-CoA carboxylase (ACC). This finding was recapitulated using primary human hepatocytes. Pretreatment of mouse hepatocytes with a small-molecule inhibitor of AMPK, Compound C (6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine), abrogated the phosphorylation of ACC following treatment with VPA. The cytochrome P450 inhibitor 1-aminobenzotriazole

blocked the VPA-stimulated phosphorylation of AMPK, suggesting a requirement for biotransformation of VPA. In line with this, treatment of hepatocytes with metabolites of VPA resulted in increased phosphorylation of AMPK/ACC as compared with VPA. Treatment of ob/ob mice with VPA for 14 days resulted in decreased liver masses, hepatic fat accumulation, and serum glucose. These results paralleled those observed in mice treated with metformin. In addition, a targeted mass spectrometry-based metabolomics assay revealed several small molecules that were differentially abundant in the serum of ob/ob mice treated with VPA as compared with vehicle-treated mice. These studies are the first to establish VPA and its metabolites as *in vitro* activators of AMPK.

Introduction

Metabolic disorders, encompassing diabetes and obesity, are an ongoing epidemic and are rapidly becoming one of the largest public health challenges. Characterized by elevated serum glucose, increased cholesterol and triglycerides, and insulin resistance, metabolic disorders result in increased risk of cardiovascular disease, hypertension, stroke, and many other health concerns. As such, there is a need for new and efficacious therapies to biochemically treat metabolic disorders. A number of drugs used for treating metabolic disorders, including metformin and thiazolidinediones, have been demonstrated to act at least in part through the activation of AMP-activated protein kinase (AMPK).

AMPK is a heterotrimeric serine/threonine kinase that has been demonstrated to play a key role in the regulation of cellular energy metabolism. In response to a reduction in cellular energy resulting from cellular or metabolic stresses, AMPK inhibits anabolic pathways that consume ATP and activates catabolic pathways that produce ATP to re-establish the cellular energy homeostasis (Kahn et al., 2005). Activation occurs via phosphorylation of the α subunit (Thr172) and subsequently phosphorylates and inactivates acetyl-CoA

carboxylase (ACC), the enzyme that catalyzes the formation of malonyl-CoA via carboxylation of acetyl-CoA, resulting in diminished biosynthesis of fatty acids and stimulation of fatty acid oxidation (Bonnefont et al., 2004). To this end, AMPK has become an attractive therapeutic target in the treatment of metabolic disorders, including type 2 diabetes and obesity (Winder and Hardie, 1999).

Valproic acid (VPA) is one of the most commonly prescribed antiepileptic drugs and has also been found effective for the treatment of bipolar disorders and migraine headaches. In addition, VPA is currently under investigation as a histone deacetylase (HDAC) inhibitor for the treatment of human immunodeficiency virus and various cancers (Nau and Loscher, 1984; Phiel et al., 2001). VPA is extensively metabolized by the cytochromes P450 and via β -oxidation, and many of its primary metabolites have been demonstrated to have antiepileptic efficacy (Nau and Loscher, 1984). Several of these metabolites of VPA, including 2-ene-VPA, 4-ene-VPA, 3-OH-VPA, and 3-keto-VPA, have been detected in the circulating plasma following dosing of VPA (Nau and Löscher, 1982; Acheampong et al., 1983). VPA has also been demonstrated to affect carbohydrate and lipid metabolism by a decreased rate of fatty acid synthesis, decreased cellular acetyl-CoA, and decreased cellular citrate (Becker and Harris, 1983). In addition, VPA has been shown to decrease the rate of glucose-6-phosphate dehydrogenase and glutathione peroxidase activity (Cotariu

This work was supported by the National Institutes of Health [Grant R01GM103853].
dx.doi.org/10.1124/mol.113.089755.

ABBREVIATIONS: ABT, 1-aminobenzotriazole; ACC, acetyl-CoA carboxylase; ALT, alanine aminotransaminase; AMPK, AMP-activated protein kinase; Compound C, 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine; FBS, fetal bovine serum; HDAC, histone deacetylase; TSA, trichostatin A; uHPLC-MS/MS, ultra-high-performance liquid chromatography–tandem mass spectrometry; VPA, valproic acid.

et al., 1990). These studies combined suggest that VPA may have an impact on the regulation of cellular metabolism.

In the present study, we demonstrate that VPA, as well as the cytochrome P450- and β -oxidation-dependent metabolites of VPA, activate AMPK in vitro, and that in vivo treatment with VPA results in significantly decreased liver mass/fat content and serum glucose in ob/ob mice. In addition, utilizing a targeted metabolomics approach, we have identified several endogenous small molecules that may be modulated in response to VPA. Targeted metabolomics is a powerful approach to probing changes in the endogenous cellular metabolome. As opposed to global metabolomics, targeted metabolomics is designed to identify a defined set of components of biochemical pathways of interest and allows for greater sensitivity and selectivity. As such, we have designed an ultra-high-performance liquid chromatography–tandem mass spectrometry (uHPLC-MS/MS) method for characterizing the levels of endogenous small molecules that play key roles in the tricarboxylic acid cycle, glycolysis, the urea cycle, the glutathione pathway, and the pentose phosphate pathway, as well as amino acids and nucleoside bases. This study is the first to define VPA as an activator of AMPK and to demonstrate the ability of VPA to decrease liver mass/fat content and serum glucose in vivo in ob/ob mice.

Materials and Methods

Chemicals and Reagents. Valproic acid sodium salt was obtained from Sigma-Aldrich (St. Louis, MO). 2-Ene-VPA, 4-ene-VPA, 3-OH-VPA, and 3-keto-VPA were obtained from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Metformin (Glucophage) and VPA (Depakote) for use in the animal study were obtained from the Johns Hopkins Hospital pharmacy supply store (Baltimore, MD). Compound C (6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine) was purchased from Calbiochem of EMD Millipore (EMD Millipore, Billerica, MA). 1-Aminobenzotriazole was purchased from Sigma-Aldrich.

Isolation of Primary Mouse Hepatocytes. Primary murine hepatocytes were isolated from 9–12-week-old male C57BL/6 mice obtained from The Jackson Laboratories (Bar Harbor, ME) via collagenase liver perfusion as previously described (Lee et al., 2004). Hepatocytes were plated in Williams' E medium supplemented with 5% fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine. Following incubation of cultures for 24 hours, medium was refreshed 4 hours prior to the treatment of hepatocytes with 800 μ M of VPA or 20 μ M of 2-ene-VPA, 4-ene-VPA, 3-OH-VPA, 3-keto-VPA or vehicle solvent (dimethylsulfoxide or water). For inhibition experiments, hepatocytes were preincubated with 10 μ M Compound C for 30 minutes (Hsu et al., 2011), and 1 mM 1-aminobenzotriazole for 1 hour prior (Bumpus, 2011).

Primary Human Hepatocytes. Primary human hepatocytes were obtained from XenoTech LLC (Lenexa, KS). Four preparations were used: male, 55 years old; female, 43 years old; female, 59 years old; and male, 36 years old. The hepatocytes had reported viabilities of 95.4, 74.7, 77.9, and 74.9%, respectively. Upon receipt, the shipping medium was changed to Williams' E medium containing 10% FBS, penicillin, streptomycin, and L-glutamine. Following 24 hours of incubation at 37°C and 5% CO₂, the media was changed to 5% FBS, penicillin, streptomycin, and L-glutamine 4 hours prior to treatments. Hepatocytes were then incubated with 800 μ M of VPA.

Immunoblot Analysis. Cells were harvested as previously described (Bumpus, 2011). Proteins (20 μ g) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrotransferred to a nitrocellulose membrane, and probed using antibodies against

AMPK α , phosphorylated (p-) AMPK α (Thr172), ACC, p-ACC (Ser79), and β -actin (obtained from Cell Signaling Technology, Danvers, MA).

HDAC Activity Assay. Analysis of HDAC activity was performed using an HDAC activity fluorometric assay kit (Cayman Chemicals, Ann Arbor, MI) and was carried out according to the manufacturer's instructions. Crude nuclear extracts were prepared from primary mouse hepatocytes as described in the manufacturer's instructions.

In Vivo Mouse Study. All treatments of mice as a part of this study were in accordance with protocols that were approved by the Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine. Mice (8–9 weeks old) homozygous for the leptin obese mutation (B6.V-*Lep^{ob}/J*; herein denoted as ob/ob mice) were purchased from The Jackson Laboratories. Metformin was used as a positive control for comparison of VPA treatment in this study, since it has been shown previously to improve the obese phenotype of ob/ob mice. Administration of VPA and metformin was carried out via dissolution in the animals' drinking water for consumption over a period of 14 days. This method has been previously used for administration of metformin and VPA (Nau and Löscher, 1982, 1984; Sugai et al., 2004; Ma et al., 2007; Phoenix et al., 2009). Body masses were recorded on day 1 prior to treatment, day 7, and day 14. After 14 days of drug treatments, all mice were euthanized by isoflurane inhalation followed by cervical dislocation, and whole blood was collected immediately. The livers were weighed immediately, rinsed in cold phosphate-buffered saline, and fixed in 10% formalin for 48 hours for hematoxylin and eosin staining.

Serum Glucose and Triglycerides. Serum glucose was analyzed using a Colorimetric Glucose Assay obtained from BioVision, Inc. (Milpitas, CA) and was performed according to manufacturer's instructions. Serum triglycerides were analyzed utilizing a Serum Triglyceride Determination Kit obtained from Sigma-Aldrich and was performed according to manufacturer's instructions.

uHPLC-MS/MS Targeted Metabolomics. Serum from each mouse was analyzed using a targeted uHPLC-MS/MS metabolomics method designed to detect endogenous biochemical pathway intermediates. The uHPLC-MS/MS assay is composed of separate positive ion and negative ion mode methods to detect 32 biochemical metabolites of the tricarboxylic acid cycle, glutathione pathway, pentose phosphate pathway, and glycolysis, as well as amino acids and nucleotides. The instrumentation was composed of a Thermo Scientific TSQ Vantage Triple Stage Quadrupole mass spectrometer interfaced with a Dionex UltiMate 3000 uHPLC system (Thermo Scientific, Waltham, MA). Optimal parent mass, product ion transitions, and collision energies were determined for each molecule using synthetic standards. Resolution and sample preparation was performed as previously described (Lade et al., 2013). Table 1 details the compounds analyzed, respective selected reaction monitoring transitions, collision energies, and retention times.

Results

VPA Stimulates Phosphorylation of AMPK and ACC in Hepatocytes. To examine the effect of VPA on AMPK phosphorylation status, we treated primary mouse hepatocytes with 800 μ M VPA over a time course of 1, 2, 4, 8, 12, and 24 hours. Phosphorylation (Thr172)/activation of AMPK was observed following 1 hour of treatment with VPA. AMPK protein levels did not change significantly as a result of VPA treatment (Fig. 1A). The phosphorylation (Ser79) of ACC, indicative of its inactivation, was also observed in the presence of VPA (Fig. 1B). ACC protein levels were not significantly different following VPA treatment. To test if the phosphorylation of AMPK and ACC was dose-dependent, primary mouse hepatocytes were treated with concentrations of VPA ranging from 200 μ M to 2 mM, and maximal phosphorylation of AMPK and ACC was observed at 800 μ M

TABLE 1

Endogenous small molecules detected using uHPLC-MS/MS

Parent mass ($[M+H]$ or $[M-H]$), product ion transitions, collision energies, and retention times were optimized for each individual compound. Note: The indicated transition for fructose/glucose-6-phosphate may be specific to either fructose-6-phosphate or glucose-6-phosphate, as the structural isomers were not able to be differentiated using uHPLC-MS/MS.

Compound	Formula Weight	$[M+H]$ or $[M-H]$	Product Ion	Collision Energy	Retention Time
	<i>g/mol</i>			<i>volts</i>	<i>minutes</i>
Positive ion mode assay					
Aspartate	133.1	134	88	13	1.15
Homocysteine	135.2	136.1	90	12	1.15
Lysine	146.2	146.9	84.1	15	0.85
Glutamate	147.1	148.1	84	14	0.97
Methionine	149.2	150	45	54	1.1
Histidine	155.2	155.8	83	28	0.92
Phenylalanine	165.2	166.1	120	11	2.61
Arginine	174.2	175.1	60	14	0.93
Citrulline	175.2	176	113	16	0.93
Tyrosine	181.2	182.1	91	28	1.34
Tryptophan	204.2	205.1	146	21	3.81
Cytidine	243.2	244.1	112	31	1
γ -Glutamylcysteine	250.3	251.1	84	22	1.24
Adenosine	267.2	268.1	136	18	2.03
Guanosine	283.2	284.1	152	14	2.05
S-Adenosylhomocysteine	384.4	385.1	136	28	1.36
Negative ion mode assay					
Succinate	118.1	116.8	73	15	1.04
Malate	134.1	132.8	71.2	20	0.86
α -Ketoglutarate	146.1	145.1	101	18	0.97
Citrate	192.1	191	110.9	13	0.98
Uridine	244.2	242.9	110	20	0.99
Fructose/glucose-6-phosphate	260.1	259	96.9	16	0.80

VPA (Fig. 1C). Circulating plasma concentrations of VPA following dosing have been reported to be 400–1000 μ M (Sztajnkrzyer, 2002; Silva et al., 2008). To determine whether phosphorylation of ACC stimulated by VPA treatment was dependent on AMPK activation, we pretreated mouse hepatocytes with a small-molecule inhibitor of AMPK: Compound C. The presence of Compound C abrogated the phosphorylation of ACC (Fig. 2).

To test whether activation of AMPK by VPA was conserved in humans, primary human hepatocytes were treated with 800 μ M VPA for 1 to 24 hours. In each of the four donors employed, phosphorylation of AMPK and ACC was observed following 1 hour of treatment with VPA (Fig. 3). Since the concentration and incubation time for stimulation of AMPK/ACC phosphorylation by VPA in primary human hepatocytes paralleled those observed using primary mouse hepatocytes we performed all subsequent studies using primary mouse hepatocytes.

Cytochrome P450-Dependent Metabolites of VPA Contribute to AMPK Activation. Since VPA is extensively metabolized by the cytochromes P450 and via β -oxidation, we sought to examine the phosphorylation of AMPK and ACC following treatment of primary mouse hepatocytes with metabolites of VPA. The cytochromes P450 have been shown to form the 4-ene-VPA and 3-OH-VPA metabolites, whereas 2-ene-VPA and 3-OH-VPA metabolites result from β -oxidation. 3-Keto-VPA is formed by dehydrogenation of the cytochrome P450 formation of 3-OH-VPA (Kiang et al., 2011). Primary mouse hepatocytes were incubated with 20 μ M of 2-ene-VPA, 4-ene-VPA, and 3-OH-VPA and compared with a vehicle-treated control (Fig. 4A). Interestingly, treatment with the metabolites of VPA resulted in levels of phosphorylated AMPK/ACC that were greater than treatment with VPA

itself, and this was most notable for 4-ene-VPA, a cytochrome P450-dependent metabolite. Treatment with a higher concentration (100 μ M) of 2-ene-VPA, 4-ene-VPA, and 3-OH-VPA resulted in levels of phosphorylated AMPK/ACC that were commensurate with those observed using 20 μ M of metabolites (data not shown). To test whether activation of AMPK by VPA required cytochrome P450-dependent metabolism, 1-aminobenzotriazole (ABT), an irreversible inhibitor of the cytochrome P450 superfamily of enzymes, was employed. Levels of phosphorylated AMPK were not elevated in samples treated with both ABT and VPA as compared with those incubated with ABT alone (Fig. 4, B and C).

HDAC Inhibition by VPA and Its Metabolites. It has been previously demonstrated in yeast and HepG2 cells (a hepatocarcinoma cell line) that the activity of AMPK is regulated by HDAC1 (Lin et al., 2012). Deacetylation of AMPK by HDAC1 was shown to facilitate its interaction with upstream kinases, thereby stimulating the phosphorylation and activation of AMPK. With these findings in mind, since VPA has been identified as an HDAC1 inhibitor (Phiel et al., 2001), it might be expected that treatment of hepatocytes with VPA would inhibit the phosphorylation/activation of AMPK via diminishing HDAC1 activity, which would be in opposition to the activation of AMPK that we have observed. Although regulation of AMPK activity by histone deacetylases has yet to be demonstrated in primary mouse or human hepatocytes, we sought to examine the HDAC inhibitory activity of both VPA and its primary metabolites and their effect on AMPK acetylation in liver/hepatocytes at the concentrations that we found increased the levels of phosphorylated AMPK/ACC.

In crude nuclear extracts prepared from mouse liver, 2-ene-VPA, 4-ene-VPA, 3-OH-VPA, 3-keto-VPA, VPA, and trichostatin A (TSA) resulted in 93.6 ± 3.6 , 90.1 ± 3.9 , 98 ± 3.9 , 94 ± 7.3 ,

Fig. 1. VPA treatment results in increased levels of phosphorylated AMPK/ACC in primary mouse hepatocytes. Primary mouse hepatocytes were treated with vehicle or 800 μ M VPA for the indicated time points and immunoblotted for p-AMPK α , AMPK α , p-ACC, ACC, and β -actin (A and B). Dose-dependence of levels of p-AMPK α and p-ACC in response to VPA were measured following 2-hour treatment with either vehicle or 200, 400, and 800 μ M, and 1.2 or 2 mM of VPA (C). Immunoblots shown in A and C are representative of four independent experiments and hepatocyte isolations.

76.4 $\pm$ 1.2, and 34.6 $\pm$ 4.3% deacetylation, respectively, as compared with samples treated with vehicle only (Fig. 5A). TSA is an established HDAC1 inhibitor and served as a positive control (Phiel et al., 2001). Using purified human recombinant HDAC1, 2-ene-VPA, 4-ene-VPA, 3-OH-VPA, 3-keto-VPA, VPA, and TSA resulted in 90 $\pm$ 9.1, 81.5 $\pm$ 15.5, 86 $\pm$ 16.8, 90.9 $\pm$ 15.2, 73.2 $\pm$ 5, and 28.9 $\pm$ 5.5% deacetylation, respectively (Fig. 5B). Inhibition of human recombinant HDAC1 activity by VPA metabolites was also not observed using concentrations ranging from 20 μ M to 2 mM (Fig. 5C).

Fig. 2. Phosphorylation of ACC following VPA treatment is AMPK-dependent. Hepatocytes were incubated with 10 μ M Compound C for 30 minutes prior to treatment with 800 μ M VPA for 2 hours and immunoblotted for p-AMPK α , p-ACC, and β -actin (A). Immunoblots shown are representative of four independent experiments and hepatocyte isolations. Densitometry analyses were performed and are reported as the mean $\pm$ S.D. of samples from four independent experiments using the treatment combinations indicated in B and C. Student's *t* tests were performed comparing the fold change of VPA versus vehicle and the fold change of Compound C + VPA versus Compound C alone to determine significance. **P* < 0.05; ***P* < 0.01. DMSO, dimethylsulfoxide.

Fig. 3. VPA treatment results in increased levels of phosphorylated AMPK/ACC in primary human hepatocytes. Primary human hepatocytes were treated with vehicle or 800 μ M VPA for the indicated time points and immunoblotted for p-AMPK α , AMPK α , p-ACC, ACC, and β -actin (A–D). Results are shown for four individual human hepatocyte preparations isolated from nonliving donors: (A) female, 59 years old; (B) female, 43 years old; (C) male, 36 years old, and (D) male, 55 years old.

Only inhibition of HDAC1 by VPA ($P < 0.001$) and TSA ($P < 0.001$) were found to be statistically significant as compared with samples treated with vehicle only; however, VPA did not completely inhibit HDAC1 as deacetylase activity was still present. VPA inhibited the deacetylase activity ($P < 0.001$) of both mouse liver nuclear extracts and human recombinant HDAC1 while of the metabolites of VPA, only 2-ene-VPA and 4-ene-VPA diminished deacetylase activity ($P < 0.05$). This was only observed using the mouse liver nuclear extracts; however, in all instances greater than 70% of the acetylation activity was remaining. In light of this, we examined the impact of VPA on AMPK acetylation status in hepatocytes via immunoprecipitation of AMPK α followed by immunoblotting for acetylated lysines (Fig. 5D). Acetylation of AMPK α in the vehicle treatment samples was commensurate with that of the samples' treatment with VPA and metabolites of VPA.

Treatment of ob/ob Mice with VPA Reduced Liver Mass/Fat Content, and Serum Glucose. Following the observation that VPA treatment of both human and mouse primary hepatocytes results in the activation of AMPK, we sought to test whether VPA may have in vivo efficacy toward improving the obese phenotype of ob/ob mice. These mice are a useful animal model for studying metabolic disorders due to the fact that they exhibit hyperglycemia, insulin resistance, fatty liver, and rapid weight gain (Drel et al., 2006). Body masses were recorded on days 1, 7, and 14 and were 50.5 ± 2.4 , 53.2 ± 2.5 , and 55.6 ± 2.7 g for the untreated mice; 45.4 ± 6 , 47.4 ± 5.6 , and 50 ± 6.1 g for the metformin-treated mice; and 50.2 ± 3 , 51.8 ± 3 , and 53.8 ± 2.7 g for the VPA-treated mice, respectively. Interestingly, only the untreated mice exhibited a significant increase in body mass from day 1 to day 14 ($P = 0.03$). Sections of each liver were also fixed and stained

with hematoxylin and eosin. The histology of the metformin- and VPA-treated groups revealed a marked reduction in the accumulation of fats in the liver as compared with the untreated mice (Fig. 6A). Excision of livers from each mouse revealed that the ratio of liver mass to body mass was significantly decreased in the metformin ($P = 0.017$) and VPA ($P = 0.004$) treatment groups as compared with untreated mice (Fig. 6B). The serum from each treatment group was analyzed for glucose concentrations, triglyceride concentrations, and alanine aminotransaminase. Serum glucose concentrations were significantly decreased in both metformin ($P = 0.01$) and VPA ($P = 0.018$) treatment groups as compared with untreated mice (Fig. 6C). Serum triglyceride concentrations were also decreased in the metformin treatment group ($P = 0.011$) and showed a trend toward a decrease in the VPA treatment group as compared with untreated mice (Fig. 6D); however, this did not reach statistical significance. Since VPA has been associated with hepatotoxicity in vivo, we measured serum activity of alanine aminotransaminase (ALT), a biomarker for hepatotoxicity. In the untreated, metformin-treated, and VPA-treated groups, ALT activity was measured at 23.8 ± 3.4 , 13.3 ± 4 , 17.7 ± 3.2 , and 17.7 ± 14.5 mU/ml, respectively (Fig. 6E). These values are all within the range of normal and indicate that VPA did not induce hepatotoxicity in these mice.

uHPLC-MS/MS-Targeted Metabolomics. An uHPLC-MS/MS assay was developed to probe for differences in the abundance of 32 endogenous small molecules using a targeted approach. Specific targeting of metabolites of interest results in greater sensitivity and selectivity as compared with detection using a global approach. The small-molecule metabolites were chosen based on their involvement in

Fig. 4. Metabolites of VPA contribute to VPA-mediated AMPK/ACC phosphorylation. Primary mouse hepatocytes were treated with either vehicle, 800 μ M VPA, or 20 μ M of 2-ene-VPA, 4-ene-VPA, 3-OH-VPA, or 3-keto-VPA for 2 hours (A). Hepatocytes were incubated with 1 mM of ABT for 1 hour prior to treatment with 800 μ M VPA for 2 hours (B). Immunoblots shown in A and B are representative of four independent experiments. Densitometry analyses were performed and are reported as the mean $\pm$ S.D. of samples from four independent experiments using the treatment combinations indicated in C. Student's *t* tests were performed comparing the fold change of VPA versus vehicle and the fold change of ABT + VPA versus ABT alone to determine significance. **P* < 0.05; ***P* < 0.01. DMSO, dimethylsulfoxide.

critical biochemical pathways involved in cellular metabolism. Of the 32 compounds screened, 22 of these compounds were detectable in the serum of the ob/ob mice. Differences in the relative levels of nine of these small molecules were

observed in the serum of the VPA-treated mice when compared with the untreated mice. Significant increases were detected for aspartate, lysine, methionine, histidine, phenylalanine, tyrosine, tryptophan, and adenosine for the VPA-treated mice. Decreased relative levels of glutamate were also noted in the VPA-treated mice as compared with the untreated control mice (Fig. 7).

Discussion

VPA has been used in therapy for decades for a multitude of disorders; however, to date it has yet to be demonstrated to activate AMPK. Using primary mouse hepatocytes, we have demonstrated that VPA treatment increases the levels of phosphorylated AMPK and ACC, and also that the phosphorylation of ACC was dependent on AMPK activity. In addition, we found that this effect was conserved in primary human hepatocytes, which showed similar time and concentration dependence. Because of the conservation of AMPK/ACC phosphorylation in mouse and human, we were able to use the primary mouse hepatocyte model in our subsequent studies.

Treatment of primary mouse and human hepatocytes with 800 μ M of VPA resulted in increased levels of phosphorylation AMPK/ACC. VPA is often dosed at 10 mg/kg/day in adults, which results in 400–1000 μ M serum concentrations (Sztajnkrzyer, 2002; Silva et al., 2008). This concentration range has been found effective for the treatment of epilepsy, migraines, and bipolar disorders (Depakote package insert; AbbVie Inc., North Chicago, IL). The time and concentration-dependence of AMPK/ACC phosphorylation in primary mouse hepatocytes following treatment with VPA is similar to that which has been reported for metformin (Foretz et al., 2010). Of note, two of the four primary human hepatocyte donors exhibited elevated levels of phosphorylated AMPK/ACC following 1 hour of treatment with either vehicle (water) or VPA, and this subsided by 4 hours. These data indicate that there may have been basal activation of AMPK at the earliest time point in these two hepatocyte preparations and not in the other donors. Phosphorylation of AMPK/ACC in primary hepatocytes was stimulated using lower concentrations of the metabolites of VPA than were required for VPA itself, indicating that the metabolites may be more potent activators of this pathway than the parent compound. This was most notable for 4-ene-VPA, a cytochrome P450-dependent metabolite. Further, the presence of the cytochrome P450 inhibitor ABT blocked the VPA-stimulated increase in the levels of phosphorylated AMPK, indicating that biotransformation of VPA is required for this effect. The metabolites of VPA evaluated in this study have been previously shown to have approximate maximal serum concentrations of 20 μ M (Nau and Löscher, 1982; Acheampong et al., 1983). With this in mind, this was the concentration employed for each of the metabolites in the present study. Interestingly, this is the first study to the best of our knowledge to test a role for drug metabolites in the activation of AMPK. While metabolism of VPA in humans versus mice may ultimately differ, the metabolites examined in this study (2-ene-VPA, 4-ene-VPA, 3-OH-VPA, and 3-keto-VPA) have been previously detected in the serum of both mice and humans following dosing of VPA (Nau and Löscher, 1982, 1984; Acheampong et al., 1983).

Fig. 5. Examination of HDAC inhibition by VPA and VPA metabolites. HDAC activity in the presence of vehicle; 800 μ M VPA; 1 μ M TSA; or 20 μ M of 2-ene-VPA, 4-ene-VPA, 3-OH-VPA, or 3-keto-VPA was assessed using crude nuclear extracts prepared from mouse hepatocytes (A) and human recombinant HDAC1 (B). Deacetylation was measured using a fluorimetric assay. Dose-dependence of VPA metabolite inhibition of human recombinant HDAC1 at 20, 100, and 500 μ M, and 1 or 2 mM for each metabolite (note: only 20 μ M–1 mM was used for 2-ene-VPA) (C). AMPK α was immunoprecipitated and immunoblotted for AMPK α and acetylated lysine (D). Immunoblots shown in D are representative of three independent experiments. Data in graphs are reported as the mean $\pm$ S.D. of samples from four independent experiments. Student's *t* tests were performed (vehicle versus VPA or metabolite-treated samples) to determine significance. **P* < 0.05; ****P* < 0.001. DMSO, dimethylsulfoxide.

Since VPA is a known inhibitor of HDAC1 (Phiel et al., 2001), which has been previously shown to regulate AMPK phosphorylation/activation (Lin et al., 2012), our data demonstrating that treatment with VPA resulted in increased levels of phosphorylated AMPK were potentially in conflict with this finding. We found that although inhibition of deacetylase activity was observed following treatment of mouse nuclear extracts and human recombinant HDAC1 with VPA, the acetylation of AMPK in primary mouse hepatocytes was unchanged following treatment with VPA as compared with vehicle treatment, indicating that any inhibition of HDAC1 activity that may have occurred as a result of VPA treatment did not impact the acetylation status of AMPK as compared with vehicle-treated control samples. Similarly, although 2-ene-VPA and 4-ene-VPA were shown to decrease the deacetylase activity of mouse nuclear extract, these metabolites did not appear to have an impact on the levels of acetylated AMPK. Of note, none of the metabolites exhibited inhibitory activity toward human recombinant HDAC1.

In addition to establishing that VPA treatment increased phosphorylation of AMPK in vitro, we found that treatment of ob/ob mice with VPA resulted in decreased liver masses, decreased lipid accumulation as determined via liver histology,

and decreased serum glucose concentrations. These outcomes were commensurate with those achieved in the metformin-treated mice. The effects of metformin on liver mass, hepatic fat accumulation, serum triglycerides, and serum glucose in vivo have been well documented (Zhou et al., 2001; Shaw et al., 2005; Foretz et al., 2010). Treatment with VPA resulted in a trend toward decreased serum triglycerides; however, it was not statistically significant compared with untreated mice. With regard to metformin, these endpoints have been previously demonstrated to be mediated by AMPK as a result of the ability of this kinase to modulate fatty acid oxidation and hepatic gluconeogenesis (Shaw et al., 2005); however, metformin has also been recently demonstrated to mediate these effects in an AMPK-independent manner (Hardie, 2013; Miller et al., 2013), revealing that there may be additional mechanisms which can contribute to the therapeutic mechanism of action for metformin, and therefore could also be considered for VPA. While VPA has been previously associated with hepatotoxicity in humans, in the present study we did not detect any abnormalities in the histology of VPA-treated livers, and ALT levels were commensurate with the normal range for ob/ob mice. The data presented here also support a previous finding that demonstrated that VPA treatment in rat hepatocytes affected

Fig. 6. Chronic administration of VPA to ob/ob mice results in decreased liver mass, hepatic lipid accumulation, and serum glucose. Ob/ob mice were administered 0.26% (w/v) VPA, 0.5% (w/v) metformin, or untreated water via their drinking water for 14 days. A section of each liver was analyzed using hematoxylin and eosin staining. Histology images are representative of four mice per treatment group (A). The mass of each liver was measured and calculated as a ratio to body mass (B). Serum glucose (C) and serum triglyceride (D) concentrations were measured using colorimetric assays. ALT was measured using a colorimetric assay to assess for hepatotoxicity (E). Data in graphs are reported as the mean $\pm$ S.D. of four mice per cohort. Student's *t* tests were performed (untreated versus VPA- or metformin-treated) to determine significance. **P* < 0.05; ***P* < 0.01.

Fig. 7. uHPLC-MS/MS-targeted metabolomics analysis of ob/ob mouse serum following chronic administration of VPA. Serum was obtained on day 14 by centrifugation of whole blood and was extracted by acetonitrile protein precipitation for uHPLC-MS/MS analysis. Of the 32 endogenous small molecules that were screened, 22 were detectable in serum, and of these, 9 endogenous metabolites exhibited significant differences among treatment groups. The relative abundance of each metabolite was detected in selected reaction monitoring mode using either positive or negative ionization. The transitions that were used for each analyte are listed in Table 1. Data in graphs are reported as the mean $\pm$ S.D. relative abundance of each metabolite for four mice per cohort. Student's *t* tests were performed (untreated versus VPA-treated) to determine significance. **P* < 0.05; ***P* < 0.01.

carbohydrate and lipid metabolism, observed by a decreased rate of fatty acid synthesis, acetyl-CoA concentration, and citrate concentration (Becker and Harris, 1983). Since VPA is approved for use in the treatment of several disorders, analyses could be performed to determine whether this drug would have particular utility in treating metabolic disorders in individuals already receiving VPA for the treatment of other pathologies, including epilepsy or bipolar disorder. It should be noted that several studies have reported a positive correlation between weight gain and insulin resistance in patients receiving a VPA regimen (Verrotti et al., 2002; Wirrell, 2003; Mania et al., 2011; Nanau and Neuman, 2013). These correlations have been established via comparison of patient populations taking VPA to patient populations receiving a different drug, or alternatively by comparing

patient populations taking VPA to healthy individuals. As such, a direct link to a molecular mechanism for VPA-associated weight gain has not been established. In addition, it has also been suggested that the disease states of epilepsy or bipolar disorder may underlie or contribute to these clinical observations (Keck and McElroy, 2003). Taken together, these studies bring to light the fact that analyses of body weight in the same individual before and after treatment with VPA may be necessary in working toward fully understanding the impact of VPA on weight gain.

The use of targeted metabolomics has the potential to provide a greater understanding of molecular consequences and action of therapies. To explore biochemical changes resulting from VPA treatment, we employed a targeted uHPLC-MS/MS-based metabolomics screen to monitor the

abundance of small-molecule metabolites in the serum of mice treated with VPA compared with the serum of untreated mice. Interestingly, we saw consistent increases in several amino acids as a result of VPA treatment compared with the untreated mice. Increased levels of both essential and nonessential amino acids (aspartate, lysine, methionine, phenylalanine, tyrosine, histidine, tryptophan) were noted, signifying increases in both amino acid production and dietary uptake in the VPA- and metformin-treated mice. In the context of AMPK activation, it is plausible that increased flux through the tricarboxylic acid cycle to stimulate the generation of ATP would result in increased formation of amino acids. Since these amino acids may be both precursors and byproduct reactions of the tricarboxylic acid cycle (Berg et al., 2002), it could also indicate increased production of amino acids for utilization in the tricarboxylic acid cycle. As AMPK activation is known to result in a decrease in ATP-consuming processes (Kemp et al., 1999) such as transcription, it is possible that the subsequent downregulation of protein synthesis would result in an accumulation of amino acids. Although these biochemical changes cannot be directly attributed to AMPK activation, the use of this metabolomics screening provides valuable insight to the chemical and biochemical changes that may result from VPA treatment.

The present study has established that the phosphorylation of AMPK/ACC is increased following VPA treatment in both mouse and human primary hepatocytes. Further, we have demonstrated marked decreases in liver mass/fat content and serum glucose in vivo in ob/ob mice in response to VPA treatment. These results paralleled those achieved in mice treated with metformin. In addition, use of a targeted mass spectrometry-based metabolomics assay revealed several small molecules with dissimilar abundance in the serum of ob/ob mice treated with VPA or metformin as compared with vehicle-treated mice. Overall, we have potentially demonstrated a novel mechanism of action for VPA.

Acknowledgments

The authors thank the Johns Hopkins Oncology Tissue Laboratory for performing histology.

Authorship Contributions

Participated in research design: Avery, Bumpus.

Conducted experiments: Avery.

Performed data analysis: Avery, Bumpus.

Wrote or contributed to the writing of the manuscript: Avery, Bumpus.

References

- Acheampong A, Abbott F, and Burton R (1983) Identification of valproic acid metabolites in human serum and urine using hexadeuterated valproic acid and gas chromatographic mass spectrometric analysis. *Biomed Mass Spectrom* **10**:586–595.
- Becker C-M and Harris RA (1983) Influence of valproic acid on hepatic carbohydrate and lipid metabolism. *Arch Biochem Biophys* **223**:381–392.
- Berg JM, Tymoczko JL, and Stryer L (2002) Biochemistry, 5th ed, W.H. Freeman and Co., New York, NY.
- Bonnefont J-P, Djouadi F, Prip-Buus C, Gobin S, Munnich A, and Bastin J (2004) Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. *Mol Aspects Med* **25**:495–520.
- Bumpus NN (2011) Efavirenz and 8-hydroxyefavirenz induce cell death via a JNK- and BimEL-dependent mechanism in primary human hepatocytes. *Toxicol Appl Pharmacol* **257**:227–234.
- Cotariu D, Evans S, Zaidman JL, and Marcus O (1990) Early changes in hepatic redox homeostasis following treatment with a single dose of valproic acid. *Biochem Pharmacol* **40**:589–593.
- Drel VR, Mashtalir N, Ilnytska O, Shin J, Li F, Lyzogubov VV, and Obrosova IG (2006) The leptin-deficient (ob/ob) mouse: a new animal model of peripheral neuropathy of type 2 diabetes and obesity. *Diabetes* **55**:3335–3343.
- Foretz M, Hébrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, Sakamoto K, Andreelli F, and Viollet B (2010) Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest* **120**:2355–2369.
- Hardie DG (2013) AMPK: a target for drugs and natural products with effects on both diabetes and cancer. *Diabetes* **62**:2164–2172.
- Hsu M-H, Savas Ü, Lasker JM, and Johnson EF (2011) Genistein, resveratrol, and 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside induce cytochrome P450 4F2 expression through an AMP-activated protein kinase-dependent pathway. *J Pharmacol Exp Ther* **337**:125–136.
- Kahn BB, Alquier T, Carling D, and Hardie DG (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**:15–25.
- Keck PE and McElroy SL (2003) Bipolar disorder, obesity, and pharmacotherapy-associated weight gain. *J Clin Psychiatry* **64**:1426–1435.
- Kemp BE, Mitchellhill KI, Stapleton D, Michell BJ, Chen Z-P, and Witters LA (1999) Dealing with energy demand: the AMP-activated protein kinase. *Trends Biochem Sci* **24**:22–25.
- Kiang TKL, Teng XW, Surendradoss J, Karagiozov S, Abbott FS, and Chang TKH (2011) Glutathione depletion by valproic acid in sandwich-cultured rat hepatocytes: Role of biotransformation and temporal relationship with onset of toxicity. *Toxicol Appl Pharmacol* **252**:318–324.
- Lade JM, Avery LB, and Bumpus NN (2013) Human biotransformation of the non-nucleoside reverse transcriptase inhibitor rilpivirine and a cross-species metabolism comparison. *Antimicrob Agents Chemother* **57**:5067–5079.
- Lee P, Peng H, Gelbart T, and Beutler E (2004) The IL-6- and lipopolysaccharide-induced transcription of hepcidin in HFE-, transferrin receptor 2-, and β 2-microglobulin-deficient hepatocytes. *Proc Natl Acad Sci USA* **101**:9263–9265.
- Lin YY, Kihl S, Suhail Y, Liu S-Y, Chou YH, Kuang Z, Lu JY, Khor CN, Lin C-L, and Bader JS et al. (2012) Functional dissection of lysine deacetylases reveals that HDAC1 and p300 regulate AMPK. *Nature* **482**:251–255.
- Ma TC, Buescher JL, Oatis B, Funk JA, Nash AJ, Carrier RL, and Hoyt KR (2007) Metformin therapy in a transgenic mouse model of Huntington's disease. *Neurosci Lett* **411**:98–103.
- Mania M, Kasradze S, and Okujava N (2011) Valproic Acid related metabolic syndrome in patients with epilepsy. *Georgian Med News* **194**:43–47.
- Miller RA, Chu Q, Xie J, Foretz M, Viollet B, and Birnbaum MJ (2013) Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* **494**:256–260.
- Nanau RM and Neuman MG (2013) Adverse drug reactions induced by valproic acid. *Clin Biochem* **46**:1323–1328.
- Nau H and Löscher W (1984) Valproic acid and metabolites: pharmacological and toxicological studies. *Epilepsia* **25** (Suppl 1):S14–S22.
- Nau H and Löscher W (1982) Valproic acid: brain and plasma levels of the drug and its metabolites, anticonvulsant effects and gamma-aminobutyric acid (GABA) metabolism in the mouse. *J Pharmacol Exp Ther* **220**:654–659.
- Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, and Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem* **276**:36734–36741.
- Phoenix KN, Vumbaca F, and Claffey KP (2009) Therapeutic metformin/AMPK activation promotes the angiogenic phenotype in the ERalpha negative MDA-MB-435 breast cancer model. *Breast Cancer Res Treat* **113**:101–111.
- Shaw RJ, Lamia KA, Vasquez D, Koo S-H, Bardeesy N, Depinho RA, Montminy M, and Cantley LC (2005) The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* **310**:1642–1646.
- Silva MFB, Aires CCP, Luis PBM, Ruiter JPN, IJlst L, Duran M, Wanders RJA, and Tavares de Almeida I (2008) Valproic acid metabolism and its effects on mitochondrial fatty acid oxidation: a review. *J Inher Metab Dis* **31**:205–216.
- Sugai F, Yamamoto Y, Miyaguchi K, Zhou Z, Sumi H, Hamasaki T, Goto M, and Sakoda S (2004) Benefit of valproic acid in suppressing disease progression of ALS model mice. *Eur J Neurosci* **20**:3179–3183.
- Sztajnkrzyer MD (2002) Valproic acid toxicity: overview and management. *J Toxicol Clin Toxicol* **40**:789–801.
- Verrotti A, Basciani F, De Simone M, Trotta D, Morgese G, and Chiarelli F (2002) Insulin resistance in epileptic girls who gain weight after therapy with valproic acid. *J Child Neurol* **17**:265–268.
- Winder WW and Hardie DG (1999) AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* **277**:E1–E10.
- Wirrell EC (2003) Valproic acid-associated weight gain in older children and teens with epilepsy. *Pediatr Neurol* **28**:126–129.
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenjk-Melody J, Wu M, Ventre J, Doeber T, and Fujii N et al. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**:1167–1174.

Address correspondence to: Dr. Namandjé N. Bumpus, Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, 725 North Wolfe St., Biophysics 307-A, Baltimore, MD 21205. E-mail: nbumpus1@jhmi.edu