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Phenylbutyrate therapy for maple syrup urine disease

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Therapy with sodium phenylacetate/benzoate or sodium phenylbutyrate in urea cycle disorder patients has been associated with a selective reduction in branched-chain amino acids (BCAA) in spite of adequate dietary protein intake. Based on this clinical observation, we investigated the potential of phenylbutyrate treatment to lower BCAA and their corresponding α-keto acids (BCKA) in patients with classic and variant late-onset forms of maple syrup urine disease (MSUD). We also performed *in vitro* and *in vivo* experiments to elucidate the mechanism for this effect. We found that BCAA and BCKA are both significantly reduced following phenylbutyrate therapy in control subjects and in patients with late-onset, intermediate MSUD. *In vitro* treatment with phenylbutyrate of control fibroblasts and lymphoblasts resulted in an increase in the residual enzyme activity, while treatment of MSUD cells resulted in the variable response which did not simply predict the biochemical response in the patients. *In vivo* phenylbutyrate increases the proportion of active hepatic enzyme and unphosphorylated form over the inactive phosphorylated form of the E1α subunit of the branched-chain α-keto acid dehydrogenase complex (BCKDC). Using recombinant enzymes, we show that phenylbutyrate prevents phosphorylation of E1α by inhibition of the BCKDC kinase to activate BCKDC overall activity, providing a molecular explanation for the effect of phenylbutyrate in a subset of MSUD patients. Phenylbutyrate treatment may be a valuable treatment for reducing the plasma levels of neurotoxic BCAA and their corresponding BCKA in a subset of MSUD patients and studies of its long-term efficacy are indicated.

INTRODUCTION

Maple syrup urine disease (MSUD; [MIM 248600]) is a classical inborn error of amino acid metabolism caused by deficiency of the mitochondrial branched-chain keto acid dehydrogenase complex (BCKDC) resulting in an accumulation of branched-chain amino acids (BCAA) (isoleucine, leucine and valine) and their corresponding branched-chain α-keto acids (BCKA) (α-keto-β-methylvalerate, α-ketoisocaproate and α-ketoisovalerate) in tissues and plasma. The disorder typically manifests with potentially lethal episodes of intoxication presenting with acute neurological deterioration, feeding problems, weight loss and a maple syrup odor to the urine (1). These episodes usually occur during states of catabolism induced by fasting or intercurrent illnesses and they result from the increase in plasma leucine concentration, whereas there is little apparent toxicity associated with increased levels of isoleucine or valine (2). Based on its severity, MSUD has

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been classified into five clinical subtypes: a 'classic' neonatal severe form, an 'intermediate' form, an 'intermittent' form, a 'thiamine-responsive' form and an 'E3-deficient with lactic acidosis' form. Although the correlation between clinical severity and degree of residual enzymatic activity is often inconsistent, the intermediate and intermittent forms are usually associated with some degree of residual activity with later clinical onset, while the classic form usually exhibits extremely low activity (3,4). Current treatment is based on dietary manipulations with protein restriction and a synthetic formula with reduced BCAA content (1). However, mental and social impairment are still present in the majority of these patients (5).

The BCKDC catalyzes the rate-limiting step in the catabolism of the BCAA. The enzyme complex consists of three catalytic components: a decarboxylase (E1) composed of two E1α and two E1β subunits, a transacylase (E2) core of 24 identical lipoate bearing subunits and a dehydrogenase (E3) existing as a homodimer (6). The subunits of the complex are encoded by four nuclear genes, synthesized in the cytosol, and imported into the mitochondria where assembly occurs (6). Mutations in the genes encoding the E1α, E1β and E2 subunits result in an MSUD phenotype, while mutations in the E3 subunit cause a more complex phenotype with lactic acidosis (7). Regulation of enzyme activity depends on the phosphorylation status of the E1α subunit that is specified by the BCKDC kinase (BDK) which inactivates the BCKDC (8) and by a mitochondrial matrix resident type 2C phosphatase (PP2Cm) that activates it (9).

We have previously reported that urea cycle patients on therapy with sodium phenylacetate/benzoate or sodium phenylbutyrate have selective BCAA deficiency despite adequate dietary protein intake (10). Data from the Urea Cycle Disorders Consortium have confirmed this finding in a large cross-sectional study (11). The mechanism responsible for this BCAA reduction is unknown, though chemical inhibition of BDK has been previously reported (12). In the present study, we have investigated the effect of phenylbutyrate in reducing blood BCAA and their corresponding BCKA in control subjects, as well as in patients with the classic and variant late-onset forms of MSUD. We also provide insight into the mechanism responsible for this effect through a series of *in vitro* and *in vivo* studies.

RESULTS

Phenylbutyrate reduces plasma BCAA and BCKA levels in both control and MSUD subjects

Three healthy control subjects were studied at baseline and after therapy with phenylbutyrate per the clinical protocol on steady-state protein intake. The analysis of BCAA showed a reduction in leucine and isoleucine in all three subjects ($P < 0.05$). The valine reduction after phenylbutyrate was statistically significant in two out of three subjects. The reduction in leucine levels with phenylbutyrate therapy ranged from 26 to 40% of the baseline levels (Fig. 1). A significant reduction in most of the BCKA was also detected (Fig. 1).

Based on these results, we enrolled five patients with classic or late-onset, intermediate form of MSUD for a trial with phenylbutyrate. Diagnosis of the classic and intermediate form was made based on onset of clinical symptoms beyond the neonatal period. The diagnosis of MSUD was confirmed biochemically based on the elevated leucine and on the presence of alloisoleucine in plasma. Enzyme assay and DNA analysis on these subjects were performed and are summarized in Table 1. Total BCKDC activity was measured using fibroblasts from the five MSUD patients. As previously reported (13), the enzyme activity measured *in situ* using cultured fibroblasts in the presence of the BDK inhibitor α-chloroisocaproic acid (CIC) did not appear to correlate with the clinical presentation because patients 1 and 5 had very low activity (3–7%) despite their clinically milder late-onset forms of the disease.

Upon treatment with phenylbutyrate, a reduction in both BCAA and BCKA was detected in three out of the five MSUD patients (patients 3 through 5) ($P < 0.05$) (Fig. 2). In these three responders, the leucine reduction ranged from 28 to 34% of the baseline levels. There was no simple correlation between the levels of residual enzymatic activity with the response of plasma BCAA and their BCKA to phenylbutyrate. Two of the responders (patients 4 and 5) carried E2 missense mutations, whereas the third responder (patient 3) carried an E1α missense mutation (Table 1).

Phenylbutyrate increases BCKDC enzyme activity *in vitro* and increases the unphosphorylated fraction of the E1α subunit *in vivo*

To confirm that the effect of the phenylbutyrate was specific for BCKDC activity (CIC added in assay), we measured the enzyme activity before and after incubation with phenylbutyrate in control fibroblasts and MSUD patients' fibroblasts. The control fibroblasts incubated for 48 h with 2 mM of phenylbutyrate showed a 1.7-fold increase in enzyme activity after incubation with phenylbutyrate (Table 2). A similar increase (1.7-fold) over baseline activity was also observed in one MSUD cell line (patient 5) consistent with the biochemical response for BCAA and BCKA in that patient. However, fibroblasts from patient 3 did not show an increase in enzyme activity over baseline levels (Table 2). Fibroblasts from patients 1, 2 and 4 were not available for the analysis. Next, we measured enzyme activity in control and patients' Epstein-Barr virus-transformed lymphoblast cell lines. Lymphoblast cell lines were available from all five MSUD patients and from two controls to measure BCKDC activity. Lymphoblasts were incubated for 48 h with and without 1 mM phenylbutyrate (a lower concentration of phenylbutyrate was used because of higher sensitivity of these cells than fibroblasts to the drug) and BCKDC activity (leucine oxidation without added CIC) was measured. As shown in Figure 3, culturing lymphoblasts with phenylbutyrate significantly enhanced leucine oxidation in controls (Fig. 3) and in all five patients' lymphoblasts (Fig. 3). Western blotting (Fig. 3) with antibodies that detect E1α, E1α-P, E2 and the branched-chain amino transferase (BCAT) isozymes revealed that the effects of phenylbutyrate on the cells are complex. The increased enzyme activity may result from the reduced phosphorylation of E1α and/or effect on activity that are independent of changes in E1α phosphorylation. Of the three patient responders, lymphoblasts from patients 4 and 5 exhibited a decrease in the phosphorylation state of E1α with no apparent change in

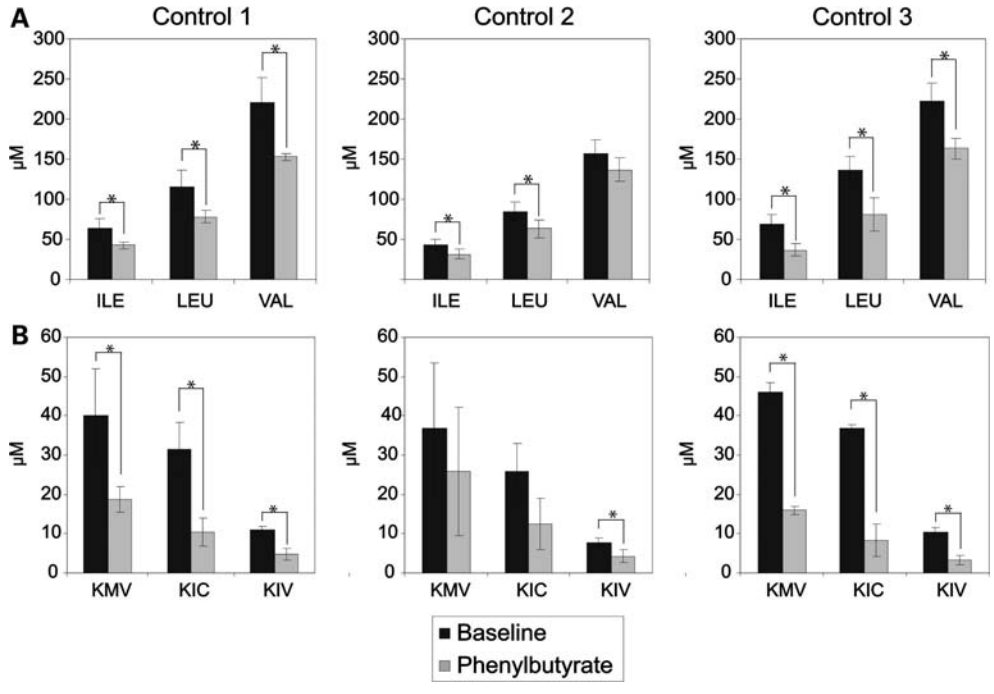

Figure 1. BCAA metabolites in control subjects. (**A**) BCAA in healthy controls before and after phenylbutyrate treatment. ILE, isoleucine; LEU, leucine; VAL, valine. $^{*}P \leq 0.05$. (**B**) BCKA in healthy controls before and after phenylbutyrate treatment. KMV, α-keto-β-methylvalerate; KIC, α-ketoisocaproate; KIV, α-ketoisovalerate. $^{*}P \leq 0.05$.

Table 1. Characteristics of the MSUD patients

	Age (years)	Gender	Fibroblast BCKDC activity[a] Mean ± SD	% of normal control	DNA analysis Affected enzyme subunit	Allele 1[b]	Allele 2[b]
Patient 1	24	Male	3 ± 2.81	0.96	E1α	p.G290R (p.G245R)[c]	p.G290R (p.G245R)[c]
Patient 2	17	Male	7.4 ± 6.7	0.9	E1α	c.887_894del[d]	p.Y438N (p.Y393N)[d]
Patient 3	5	Female	2 ± 0.74	0.26	E1α	p.V412M (p.V367M)[e]	p.V412M (p.V367M)[e]
Patient 4	6	Female	272 ± 31	36.1	E2	c.75_76del[f]	p.R301C (p.R240C)[g]
Patient 5	16	Female	7 ± 1	1.62	E2	p.S366P (p.S305P)[h]	Exon 11 del[h]

[a]Enzyme activity measured on fibroblasts in the presence of CIC expressed in pmol CO_2 released/mg protein/hour.
[b]The numbering systems of amino acid residues beginning with the initiation Methionine as +1 or with the amino terminus (in parenthesis) are both listed.
[c]This mutation was previously reported in homozygous state by Chuang *et al*. (44) in patients with an intermediate form of MSUD.
[d]Mutations previously reported by Zhang *et al*. (45) and Chuang *et al*. (46) in Mennonite patients with classic MSUD.
[e]Mutations previously reported by Henneke *et al*. (47) in patient with classic MSUD.
[f]Mutation previously reported by Fisher *et al*. (48) in compound heterozygous state with the p.E163X mutation in a patient with classic MSUD.
[g]Mutations previously reported by Brodtkorb *et al*. (49) in thiamine–responsive and intermittent MSUD patients, respectively. The R301C allele is common in heterozygous Norwegian MSUD patients.
[h]Mutations not previously reported.

E1 levels. However, very little E1α was phosphorylated in cells from patients harboring E2 mutations. E1α phosphorylation was not decreased in cells from the normal controls nor patients 1, 2 or 3 (Fig. 3). There were small variations in E2 enzyme levels between the patient and control cells (Fig. 3). Phenylbutyrate appeared to affect the levels of the BCAT isozymes, particularly the cytosolic isozyme BCATc which is expressed in lymphoblasts (and in fibroblasts, unpublished data). BCATc levels increased in response to phenylbutyrate in all of the lymphoblasts with the exception of patient 5 lymphoblast cells.

In summary, E1α exhibited little phosphorylation that was decreased further by phenylbutyrate treatment in cells from the two patients with E2 mutations (patients 4 and 5, see Table 1). In the other patient cell lines, enhanced activity did not appear to correlate with changes in phosphorylation but may have been influenced by substrate availability due to increased BCATc activity and/or direct effects on enzyme activity. Changes in BCAT isozyme activity could also impact on BCKA substrate delivery. All patient cell lines accumulated more α-ketoisocaproate than observed in control cell lines (data not shown).

To investigate the effect of phenylbutyrate *in vivo* on BCKDC, wild-type mice ($n = 5$) were given saline or phenylbutyrate orally and after 3 days of treatment they were sacrificed for analyses. The western blot analysis on the liver

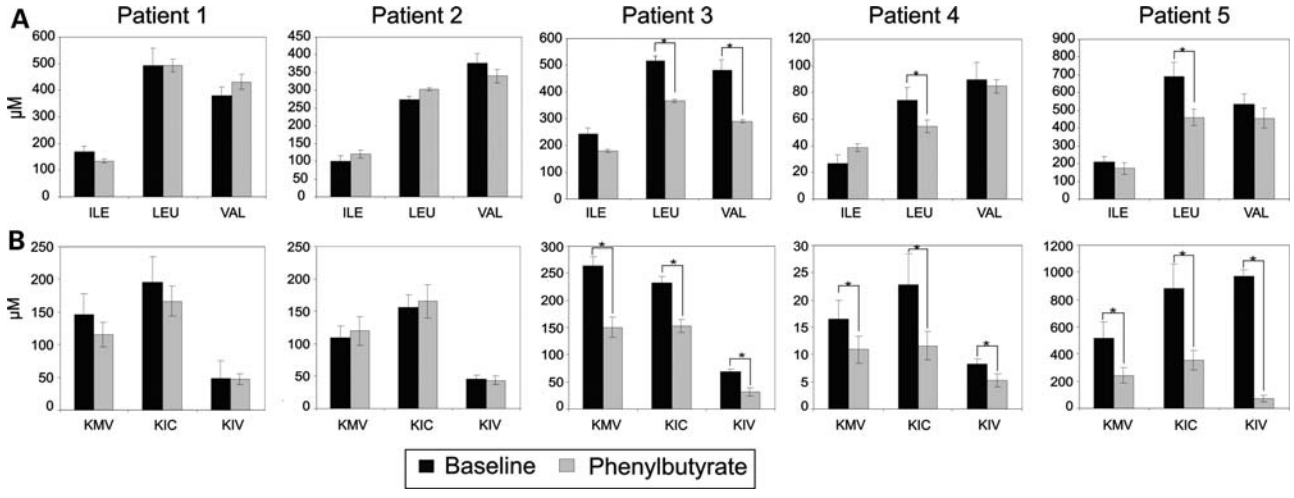

Figure 2. BCAA metabolites in MSUD subjects. (**A**) BCAA in MSUD patients before and after phenylbutyrate treatment. ILE, isoleucine; LEU, leucine; VAL, valine. $^{*}P \leq 0.05$. (**B**) BCKA in MSUD patients before and after phenylbutyrate treatment. KMV, α-keto-β-methylvalerate; KIC, α-ketoisocaproate; KIV, α-ketoisovalerate. $^{*}P \leq 0.05$.

Table 2. Percentage of normal enzyme activity before and after phenylbutyrate in skin fibroblasts[a]

	Fibroblasts	
	−	+
Normal control	100%	176.08%
Patient 1	N.A.	N.A.
Patient 2	N.A.	N.A.
Patient 3	0.59%	0.52%
Patient 4	N.A.	N.A.
Patient 5	4.47%	7.62%

[a]Enzyme activity measured on fibroblasts using the radioactive method previously described (34). N.A., not available.

extract showed that the phenylbutyrate treatment resulted in a significant reduction in the levels of the phosphorylated E1α subunit of BCKDC when compared with the saline-treated mice (Fig. 4). As shown in Figure 4, E1α and E2 protein levels were not increased by phenylbutyrate treatment, suggesting no change in BCKDC concentrations. Nevertheless, because of reports of phenylbutyrate activity as a histone deacetylase inhibitor (14), we evaluated BCKDC subunits and BDK RNA levels to determine whether increased transcription of the respective subunits could contribute to elevated enzyme levels. We found levels of E1α, E1β, E2 and E3 unchanged in treated versus untreated mouse muscle and actually decreased subunit RNA levels in treated livers (Supplementary Material, Fig. S1). These results suggest that the primary effect of phenylbutyrate is on enzyme activity and BDK-mediated phosphorylation of E1α, and not mediated by increased RNA expression of these genes.

Phenylbutyrate stimulates E1 activity, inhibits E1α phosphorylation by BDK and enhances BCKDC overall activity

To determine the effect of phenylbutyrate on individual enzymes in the BCAA catabolic pathways, activities of

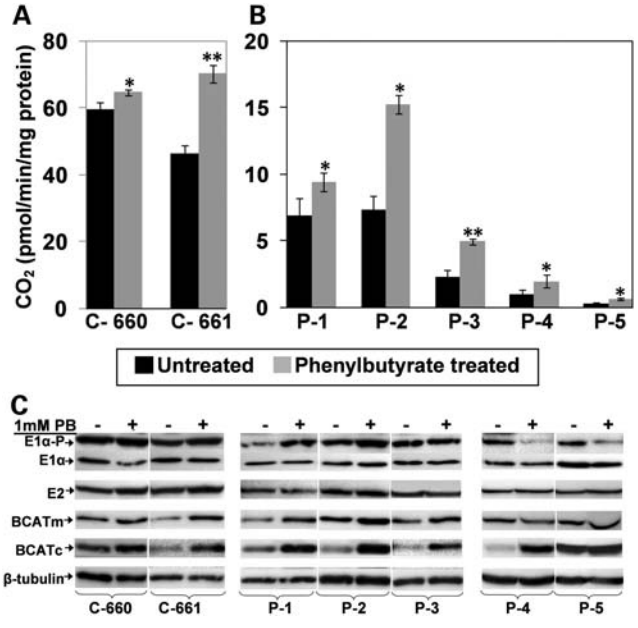

Figure 3. BCKDC activity and phosphorylation status in lymphoblasts. (**A** and **B**) BCKDC activity in lymphoblast cells from control subjects (C-660, C-661) and MSUD patients (P-1, -2, -3, -4, -5) untreated or treated with 1 mM phenylbutyrate for 48 h. Leucine oxidation was measured by using radioactive assay as described in Materials and Methods. Rates are expressed as pmol of CO_2 released/min/mg protein. Values are means ± SD ($n = 3$), * $P \leq 0.05$, $^{**}P \leq 0.01$. (**C**) Western blotting of lymphoblast cell BCKDC enzymes (E1α- P, E1α and E2), BCATm and BCATc untreated or treated with 1 mM phenylbutyrate (PB) for 48 h. β-Tubulin was used as an internal control. Images are representative of three independent experiments.

BCATm and BCKDC enzymes were measured with and without phenylbutyrate using purified recombinant enzymes. BCATm generates the BCKA products that are elevated in MSUD and are the substrates for BCKDC. As shown in Table 3, there was no effect of phenylbutyrate on BCATm kinetics including k_{cat} and K_m. BCKDC has multiple enzyme activities and is inactivated by BDK (15). Therefore, we

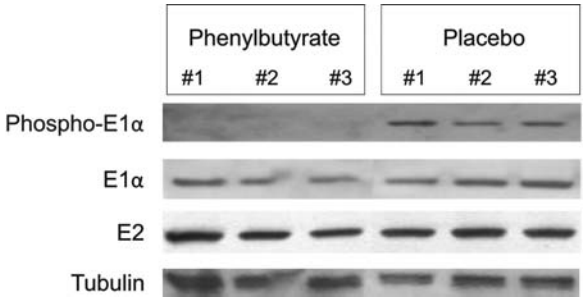

Figure 4. *In vivo* analysis of BCKDC phosphorylation status. Western blot analysis of liver extract using an antibody against the phosphorylated form of the E1α from three representative mice treated with phenylbutyrate or saline. Each lane corresponds to the liver extract from an independent mouse (from #1 to #3). The phosphorylated form of the E1α is significantly reduced in the phenylbutyrate treated mice when compared with the placebo group. The two groups showed similar amount of tubulin, E1 and E2 proteins.

assayed for the following enzyme activities in the presence and absence of phenylbutyrate: E1 activity of both unphosphorylated (fully active) and completely phosphorylated (inactive) E1, and the ability of BDK to inactivate E1. As shown in Table 4, the addition of 1 mM phenylbutyrate augmented unphosphorylated E1 (fully active enzyme)-catalyzed decarboxylation of all three BCKA substrates significantly. Phenylbutyrate did prevent inactivation of E1 in the presence of BDK; however, it did not have an effect on E1 that had been inactivated previously by BDK (Table 4, compare k_{cat} and K_m values for phosphorylated E1 versus E1 plus phenylbutyrate and BDK). Phenylbutyrate not only increased the k_{cat} (2–3-fold) but also boosted the sensitivity of the enzyme to BCKA by lowering their K_m values (38–47%). Phenylbutyrate did prevent inactivation of E1 in the presence of BDK; however, it did not have an effect on E1 that had been inactivated previously by BDK (Table 4, compare k_{cat} and K_m values for phosphorylated E1 and E1 plus phenylbutyrate and BDK). Phenylbutyrate enhanced overall BCKDC activity as shown by the increases of 50–70% in k_{cat} values for BCKA (Table 5). These results suggest that phenylbutyrate has multiple effects on BCKDC. It can enhance E1 decarboxylase activity [decarboxylation is not rate-limiting in the overall reaction of the BCKDC complex (16)], and overall BCKDC activity as well as protect BCKDC from inactivation by BDK.

To investigate whether phenylbutyrate directly inhibits BDK activity as suggested by Paxton and Harris (12), BDK-catalyzed incorporation of ^{32}P from [γ-^{32}P]ATP into E1 was determined in the presence of increasing concentrations of phenylbutyrate (Fig. 5). Phenylbutyrate inhibits BDK activity with an IC_{50} of 29.9 μM. The relatively high IC_{50} value is consistent with the high dosages required for phenylbutyrate treatments in the patient studies (10 g/m^2/day). Interestingly, the binding affinity (represented by the dissociation constant K_d) of BDK for phenylbutyrate, as determined by isothermal titration calorimety (ITC), is 6.93 ± 0.71 μM ($n = 3$). The enthalpy change (ΔH) of -7.69 ± 1.28 kcal/mole indicates the relative robustness of the BDK and phenylbutyrate interaction. The K_d value for the binding of phenylbutyrate to BDK is not affected by the addition of nucleotide ATP or ADP (data not shown). The micromolar binding affinity provides direct evidence that phenylbutyrate acts on BDK. The ITC data further show that phenylbutyrate binds to a site distinct from the ATP-binding pocket of BDK.

DISCUSSION

The results presented in this study suggest that phenylbutyrate is a potential adjunctive treatment for selected classes of MSUD patients. The ability of phenylbutyrate to enhance residual flux through the BCKDC pathway by altering the phosphorylation status of the E1α subunit as well as to directly increase E1 enzyme activity is a new and novel finding. In three patients with clinically, late-onset forms of MSUD, phenylbutyrate treatment reduced the blood concentrations of BCAA and their corresponding BCKA. Recent reports suggest that the BCKA, particularly the α-keto acid of leucine, are the toxic metabolites in MSUD (17,18). Until now, no pharmacological treatment for MSUD was available and acute decompensation due to leucine intoxication could only be treated with supportive measures and/or hemodialysis (1).

The two patients with E2 mutations (patients 4 and 5) responded to phenylbutyrate treatment with significant reductions in plasma leucine and all three BCKA. It is likely that in both of these patients, activation of E1 through the inhibition of BDK activity by phenylbutyrate increased BCKDC flux which enhanced the clearance of BCKA. On the other hand, the patient carrying the homozygous mutation p.V412M (patient 3) responded to phenylbutyrate, whereas the patient found to be a compound heterozygous for the c.887_894del and p.Y438N mutations (patient 2) did not respond. E1 is a heterotetramer of two E1α and two E1β subunits which assemble in the active enzyme. Mutation or deletion of functionally important residues might abort the tetrameric assembly. According to protein structural modeling, the V412 is at the surface accessible area of E1α to form heterotetrameric assembly with E1β. Mutations affecting this residue hamper the assembly of the complex. Addition of phenylbutyrate might stabilize assembly formation and ultimately enhance the oxidation of BCKA. A naturally occurring osmolyte trimethylamine N-oxide has been shown to correct tetrameric assembly defects caused by the Y438N mutation, leading to a partial restoration of E1 activity (19). The amino acids G290, Y438, and the residues encoded by c.887–895 are localized in the surface accessible area of E1α to form an $\alpha_2\beta_2$ tetrameric assembly with the E1β subunit. Mutations of the G290 by Arg and Y438 by Asn (20) and deletion of c.887–895 diminish the assembly formation of E1 and ultimately the total function of E1 catalyzed decarboxylation.

Both patients with E2 mutations and some residual activity responded to phenylbutyrate. The E2 reaction is considered rate-limiting for the overall BCKDC activity (21). However, the present result suggests that increasing E1 activity can increase BCKDC activity and/or activation of free E1 increases decarboxylation of BCKA sufficiently to reduce BCKA levels. Protein structural analysis shows that the amino acids R301 and S366, detected in patients 4 and 5, respectively, are among the residues involved in CoA binding (22) and core formation in 24-meric assembly. Mutations of these residues do not show significant differences in the model structure, and may be involved in overall transacylation reaction of E2.

Table 3. Phenylbutyrate had no effect on BCATm activity

Additions	BCATm transaminase activity k_{cat} (s^{-1})			K_m (mM)		
	Leu	Ile	Val	Leu	Ile	Val
[−] Phenylbutyrate	340 ± 9	380 ± 13	285 ± 16	1.7 ± 0.2	1.3 ± 0.3	8.1 ± 1
[+] Phenylbutyrate	332 ± 14	372 ± 19	296 ± 21	1.7 ± 0.3	1.3 ± 0.3	7.9 ± 1

Leu, leucine; Ile, isoleucine; Val, valine.

Table 4. Phenylbutyrate protected E1 from BDK induced inactivation and had no effect on the activity of phosphorylated E1

Additions	k_{cat} (min^{-1})			K_m (μM)		
	KIC	KMV	KIV	KIC	KMV	KIV
E1 catalyzed decarboxylase activity						
[−] Phenylbutyrate	7.6 ± 1.0	5.2 ± 0.8	12.0 ± 1.2	39.0 ± 2.0	45.0 ± 4.0	48.0 ± 3.0
[+] Phenylbutyrate	20.2 ± 1.5	18.0 ± 1.0	25.0 ± 2.0	24.0 ± 2.0	21.0 ± 3.0	22.0 ± 2.0
[+] Phenylbutyrate[a], [−] BDK	19.8 ± 1.7	20.0 ± 1.1	28.0 ± 1.9	27.0 ± 2.0	18.0 ± 2.0	20.0 ± 3.0
[+] Phenylbutyrate[a], [+] BDK	20.6 ± 2.5	22.0 ± 1.8	26.0 ± 2.2	21.0 ± 1.9	21.0 ± 3.0	25.0 ± 2.0
E1 catalyzed decarboxylase activity measured after inactivation by BDK[b]						
[−] Phenylbutyrate[b]	0.9 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	532.0 ± 35.0	610.0 ± 28.0	680.0 ± 20.0
[+] Phenylbutyrate[b]	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	550.0 ± 27.0	642.0 ± 38.0	720.0 ± 47.0

KMV, α-keto-β-methylvalerate; KIC, α-ketoisocaproate; KIV, α-ketoisovalerate.
[a]E1 protein was reconstituted with phenylbutyrate (1.0 mM) first and then BDK (0.1–0.5 μg) and ATP (0.4–1.0 mM) were added.
[b]E1 protein was phosphorylated first with the addition of BDK (0.1 μg) and ATP (0.4 mM).

Table 5. Phenylbutyrate enhanced overall BCKDC activity

Additions	Activity of BCKDC[a] k_{cat} (min^{-1})			K_m (μM)		
	KIC	KMV	KIV	KIC	KMV	KIV
[−] Phenylbutyrate	140.0 ± 15.0	118.0 ± 10.0	197.0 ± 12.0	45.0 ± 6.0	53.0 ± 7.0	55.0 ± 4.0
[+] Phenylbutyrate	255.0 ± 10.0	226.0 ± 18.0	309.0 ± 15.0	41.0 ± 5.0	50.0 ± 3.0	40.0 ± 5.0

KMV, α-keto-β-methylvalerate; KIC, α-ketoisocaproate; KIV, α-ketoisovalerate.
[a]The overall assay was measured by NADH production monitored at 340 nm. The molar ratio of recombinant E1, E2 and E3 was maintained at 12:1:55 and the activity determined at pH 8.0 and 303 K (41).

The response to phenylbutyrate is complex and may not be simply correlated with residual BCKDC activity measurements in fibroblasts or with the genotype. The enzymatic activity of BCKDC in patient fibroblasts is known to poorly correlate with the clinical severity (13). Moreover, the estimates of enzyme activity *ex vivo* using cultured patients' cell lines can be considerably different from estimates of enzyme activity *in vivo* (23). Still, in-depth structural analysis and modeling of phenylbutyrate interaction to this enzyme complex may eventually better help predict genotype–response correlations. Until more patients with a wider range of mutations have been examined, *in vivo* loading test may be required to predict phenylbutyrate responsiveness in MSUD patients.

The availability of a novel therapeutic approach to reduce the blood levels of the BCAA and their BCKA may allow for less stringent dietary restrictions as well as a potential treatment during acute metabolic decompensations. The catabolism of BCAA is tightly regulated by the kinase and phosphatase action on the E1α subunit of the E1 decarboxylase of BCKDC.

Finally, we have described a novel mechanism of phenylbutyrate action *in vivo*, which is mediated by direct BDK inhibition. A wide range of biological activities have been attributed to phenylbutyrate. In its only FDA approved use in urea cycle disorders, phenylbutyrate acts as a pro-drug leading to the generation of phenylacetate. Here, phenylacetate conjugates glutamine and serves as an alternative route of nitrogen disposal. In addition to this application, phenylbutyrate has been studied for cancer, cystic fibrosis, thalassemia, spinal muscular atrophy, amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease and type 2 diabetes mellitus (14,24–30). Biochemical activities that have been attributed in these scenarios include action as chaperone, histone deacetylase inhibitor, growth inhibition and relief of endoplasmic reticulum stress. However, the mechanistic

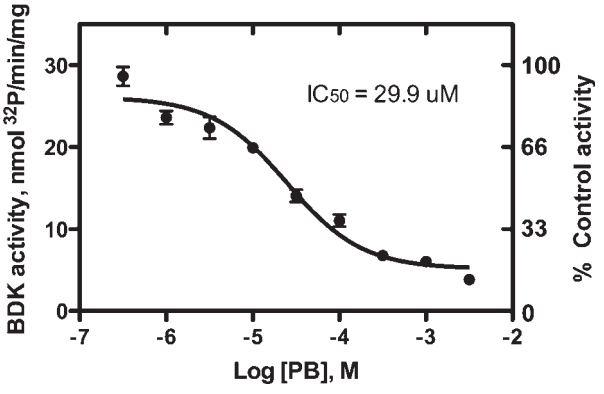

Figure 5. The IC_{50} value for inhibition of BDK by phenylbutyrate. The 100% control activity represents BDK activity in the absence of phenylbutyrate.

basis for these activities remains poorly defined. Our data suggest a novel direct effect on BCKDC via action on protein phosphorylation. As a major regulatory mechanism of almost of all biological processes, a potential approach for targeting protein phosphorylation may offer new treatment avenues in disease processes where phosphorylation is central to pathogenesis (24,26,31).

MATERIALS AND METHODS

Clinical protocol

The clinical protocol was approved by the Human Subjects Institutional Review Board of the Baylor College of Medicine. The healthy control subjects (two females and one male; of 24, 25 and 39 years of age, respectively) and the MSUD patients were admitted into the Texas Children's Hospital General Clinical Research Center and were started on the study protocol after informed consent was obtained. Each subject or a parent for those younger than 18 years gave written informed consent for participation in the study.

Both the healthy controls ($n = 3$) and the MSUD patients ($n = 5$) were admitted twice in the clinical research center for 3 days each time. For both admissions, the subjects received a constant protein intake of 0.6 g/kg/day as a combination of BCAA-free formula and whole protein. On day 3 of admission, the patient had blood sampling at 0, 4, 6 and 8 h during a period of frequent every 2 h feeds in which one-eighth of the day's protein subscription was given. On the second admission, each subject was given sodium phenylbutyrate (Buphenyl) at a dose of 10 g/m^2/day divided into four equal doses. Otherwise, blood sampling was performed in the fed state on day 3 as in the baseline admission. Plasma samples were analyzed for amino acids and their corresponding BCKA: α-keto-β-methylvalerate (KMV), α-ketoisocaproate (KIC) and α-ketoisovalerate (KIV). The concentration of the plasma amino acids was measured with the amino acid analyzer method. Plasma BCKA were derivatized with *o*-phenylenediamine and separation was made by gradient elution from a Spherisorb^{TM} ODS2 column (250 mm × 4.6 mm, 5 μm; Waters) according to protocols previously described (32,33).

Enzymatic and DNA studies

BCKDC enzyme activity was measured on skin fibroblasts from all five patients using the radioactive method previously described (34). In this method, cultured fibroblasts cells are incubated with α-1-^{14}C-leucine for 4 h in the medium to stimulate the BCKDC activity. At the end of the incubation, the amount of $^{14}CO_2$ released from leucine decarboxylation is captured onto damped filter paper. Decarboxylation activity of BCKDC is expressed as pmol of CO_2 released/mg protein/hour and as percentage of normal activity. BDK activity was assayed by the incorporation of ^{32}P-phosphate from [γ-^{32}P]ATP to the E1α subunit as described previously (35).

DNA samples from the five patients were analyzed for mutations in the *BCKDHA*, *BCKDHB* and *DBT* genes by sequencing all the coding exons and their flanking intronic regions. When only one mutation was found by sequencing, the DNA samples were further analyzed by targeted array comparative genomic hybridization to rule out intragenic deletions and duplications.

In vivo mouse studies

Mouse studies were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, Houston, TX, USA. Sodium phenylbutyrate or saline was given orally to C57B6 mice ($n = 5$ mice per group) by gavage at the dose of 50 mg/kg/day divided into three administrations for three consecutive days. After 3 days of treatment, the animals were sacrificed to harvest the livers. Proteins extracted from mouse livers were homogenized in a buffer containing 5% SDS and 0.0625 M Tris–HCl. Western blot analyses were performed using an anti-Phospho E1 antibody (36), the anti-E1 antibody (37) and the anti-E2 antibody (Kamiya Biomedical Company, Seattle, WA).

Cell studies

To study the effect of the phenylbutyrate on BCKDC activity, control and fibroblasts from two MSUD patients (patients 3 and 5) enrolled in the study were cultured in the presence or absence of phenylbutyrate (Buphenyl) at the concentration of 2 mM for 48 h. Fibroblasts for the other patients were not available for this analysis. After 48 h of incubation, the cells were washed with PBS and harvested to measure BCKDC enzymatic activity as described above. Lymphoblast cell lines were available from all five patients and were treated with phenylbutyrate at the concentration of 1 mM for 24 h. Lymphoblast cells were grown in RPMI media (Thermo Scientific HyClone, Logan, UT) with glutamine and 15% FBS for 48 h with or without 1 mM phenylbutyrate, washed twice with Krebs buffer (20 mM HEPES, pH 7.0, 128 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 5.5 mM glucose). For the leucine oxidation assay, the lymphoblast cells were incubated in Krebs buffer (without 1 mM CIC), 10 mM leucine and [1-^{14}C]- Leu (specific activity around 200 dpm/nmole). The assay was carried out in a 20 ml scintillation vial and cells were gassed with a 95% O_2:5% CO_2 mixture for 20 s sealed with a rubber stopper and incubated in Dubnoff metabolic shaker at 37°C for

120 min. The reaction was stopped by addition of 3% perchloric acid and $^{14}CO_2$ trapped and quantified as described previously (38,39). Branched-chain ^{14}C-α-keto acid production from leucine (α-ketoisocaproate) was measured as described previously (38).

Lymphoblast pellets were resuspended in extraction buffer (25 mM HEPES, pH 7.0, 0.4% CHAPS, 1 mM DTT) containing 10 mM EDTA, 10 mM EGTA, 50 mM benzamidine, 0.5 mM microstatin, 33 mM β-glycerophosphate, 0.5 mM ThDP, 1 mM sodium orthovanadate, 7.2 mM KF and sonicated. Protein was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Protein Products, Logan, UT). Proteins (50 μg) in the cell extracts were separated using SDS–PAGE and transferred to Hybond-P membranes (Amersham, Pharmacia Biotech, Piscataway, NJ). Membranes, blocked with 5% milk in 1× TBST buffer and incubated overnight with antibodies against E1α, phosphorylated E1α (E1α-P), E2, BCATm, BCATc or β-tubulin (Cell Signaling Technology, Danvers, MA); antibodies dilutions were 1:20 000, 1:15 000, 1:80 000, 1:40 000, 1:30 000 or 1:7000 for E1α, phosphorylated E1α (E1α-P), E2, BCATm, BCATc or β-tubulin, respectively. Proteins were detected using horseradish peroxidase-conjugated secondary rabbit antibodies diluted between 1:15 000 and 1:30 000 times and a chemiluminescence reagent (Pierce Protein Products, Logan, UT) following an X-ray film exposure for 10 s with the following exception. The E1α-P protein in patients 4 and 5 was in much lower amount than in the rest of the patients or controls. Therefore, the Hybond-P membrane of patients 4 and 5 was exposed for longer time (10 min) as opposed to 10 s exposure time for the rest of the patients and/or controls.

Biochemical studies

The activity of the BCATm was measured at pH 8.0 and 298 K as described previously (40). Kinetics of the E1 decarboxylase reaction with and without phenylbutyrate were determined in the presence of an artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) as reported previously (41). The assay mixture contained 21 μg of E1 in 100 mM potassium phosphate, pH 7.5, 2.0 mM $MgCl_2$, 0.2 mM thiamin diphosphate (ThDP), 0.1 mM DCPIP and 1 mM BCKA. The rate of decarboxylation at 30°C was measured by monitoring the reduction of the dye at 600 nm (42,43). For the overall BCKDC activity assay, the enzymes were exchanged into phosphate buffer (30 mM potassium phosphate, pH 7.5) containing 5 mM DTT using a PD-10 column and the enzyme concentrations were calculated from the absorption maxima at 280 nm (40). The protein complex was reconstituted with E1, lipoylated E2 (lip-E2) and E3 at a molar ratio of 12:1:55, in which lip-E2 exists as a 24-mer. The assay mixture contained 30 mM potassium phosphate pH 7.5, 100 mM NaCl, 3 mM NAD^+, 0.4 mM CoA, 2 mM $MgCl_2$, 2 mM DTT, 0.1% Triton X-100 and 2 mM ThDP. The overall reaction was monitored by the formation of NADH at 340 nm. The apparent rate constants (k_{app}) at different substrate concentrations for all of the above assays were determined from the absorption changes at the individual wavelength maximum. The k_{app} rate constants were fit using the following equation:

$$k_{app} = \frac{k_{cat}[S]}{K_m + [S]}$$

The phosphorylation of E1 was carried out in the phosphorylation reaction mix (30 mM HEPES, pH 7.4, 2 mM DTT, 1.5 mM $MgCl_2$ and 0.2 mM EGTA) with and without addition of phenylbutyrate. E1, E2 and E3 proteins were mixed at 12:1:55 molar ratio in a 0.1 ml reaction mix, and 0.1 μg of maltose-binding protein-tagged rat BDK was added. The mix was pre-incubated at room temperature for 15 min. The phosphorylation reaction was started after addition of 0.4 mM ATP to the reaction mix, and the reaction was terminated at different time points by addition of higher salt concentration. Overall, BCKDC activity was measured as described above.

Isothermal calorimetry

Maltose-binding protein-tagged BDK (MBP-BDK) was dialyzed against 50 mM potassium phosphate buffer (pH 7.5) containing 50 mM potassium chloride and 2 mM $MgCl_2$. To measure binding affinities of MBP-BDK for phenylbutyrate, 300 μM phenylbutyrate in the syringe was injected into the reaction cell containing 30 μM MBP-BCK (based on monomer) at 15°C in a VP-ITC microcalorimeter (MicroCal, Northampton, MA, USA). Dissociation constants were calculated with Origin version 7.0 software (OriginLab Corp, Northampton, MA, USA).

Protein structural analysis

Structural analyses of E1 and E2 proteins were performed using the three-dimensional structures of these proteins from protein data bank (PDB numbers 2BFE for E1 and 2II5 for E2). The data were loaded in Swiss-Pdb viewer software (Swiss Institute of Bioinformatics) and analysis of residues and their role in the complex were performed by either localization or point mutation of these residues. Thermal factors (B-factors) were used to understand the stability of the residues in the wild-type or mutant proteins.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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