Phenylbutyrate therapy for maple syrup urine disease

Nicola Brunetti-Pierri1,†, Brendan Lanpher1,‡, Ayelet Erez1, Elitsa A. Ananieva3, Mohammad Islam3, Juan C. Marini4, Qin Sun1, Chunli Yu5, Madhuri Hegde5, Jun Li6, R. Max Wynn6, David T. Chuang6, Susan Hutson3 and Brendan Lee1,2,*

1Department of Molecular and Human Genetics and 2Howard Hughes Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA, 3Human Nutrition, Foods and Exercise, Virginia Tech, 338 Wallace Hall (0131), Blacksburg, VA 24061, USA, 4United States Department of Agriculture/Agriculture Research Service Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, 1100 Bates Street, Houston, TX 77030, USA, 5Department of Human Genetics, Emory University School of Medicine, 615 Michael Street, Suite 301, Atlanta, GA 30033, USA and 6Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9038, USA

Received September 12, 2010; Revised November 10, 2010; Accepted November 17, 2010

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-ketoisocaproat 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

†These authors contributed equally to this study.

*To whom correspondence should be addressed. Tel: +1 713798 8835; Fax: +1 7137985169; Email: blee@bcm.tmc.edu

© The Author 2010. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
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 alloisoalsoleucine 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
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 1.** BCAA metabolites in control subjects. (A) BCAA in healthy controls before and after phenylbutyrate treatment. ILE, isoleucine; LEU, leucine; VAL, valine. *P ≤ 0.05. (B) BCKA in healthy controls before and after phenylbutyrate treatment. KMV, α-keto-β-methylvalerate; KIC, α-ketoisocaproate; KIV, α-ketoisovalerate. *P ≤ 0.05.

**Table 1.** Characteristics of the MSUD patients

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Gender</th>
<th>Fibroblast BCKDC activity a</th>
<th>DNA analysis</th>
<th>Affected enzyme subunit</th>
<th>Allele 1 b</th>
<th>Allele 2 b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>24</td>
<td>Male</td>
<td>3 ± 2.81</td>
<td>0.96</td>
<td>E1α</td>
<td>p.G290R (p.G245R)c</td>
</tr>
<tr>
<td>Patient 2</td>
<td>17</td>
<td>Male</td>
<td>7.4 ± 6.7</td>
<td>0.9</td>
<td>E1α</td>
<td>c.887_894deld</td>
</tr>
<tr>
<td>Patient 3</td>
<td>5</td>
<td>Female</td>
<td>2 ± 0.74</td>
<td>0.26</td>
<td>E1α</td>
<td>p.V412M (p.V367M)d</td>
</tr>
<tr>
<td>Patient 4</td>
<td>6</td>
<td>Female</td>
<td>272 ± 31</td>
<td>36.1</td>
<td>E2</td>
<td>c.75_76delf</td>
</tr>
<tr>
<td>Patient 5</td>
<td>16</td>
<td>Female</td>
<td>7 ± 1</td>
<td>1.62</td>
<td>E2</td>
<td>p.S366P (p.S305P)h</td>
</tr>
</tbody>
</table>

aEnzyme activity measured on fibroblasts in the presence of CIC expressed in pmol CO2 released/mg protein/hour.

bThe numbering systems of amino acid residues beginning with the initiation Methionine as +1 or with the amino terminus (in parenthesis) are both listed.

cThis mutation was previously reported in homozygous state by Chuang et al. (44) in patients with an intermediate form of MSUD.

dMutations previously reported by Zhang et al. (45) and Chuang et al. (46) in Mennonite patients with classic MSUD.

Mutations previously reported by Henneke et al. (47) in patient with classic MSUD.

Mutations previously reported by Fisher et al. (48) in compound heterozygous state with the p.E163X mutation in a patient with classic MSUD.

Mutations previously reported by Brodkorb et al. (49) in thiamine–responsive and intermittent MSUD patients, respectively. The R301C allele is common in heterozygous Norwegian MSUD patients.

Mutations not previously reported.
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.

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

<table>
<thead>
<tr>
<th>Fibroblasts</th>
<th>–</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>100%</td>
<td>176.08%</td>
</tr>
<tr>
<td>Patient 1</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Patient 2</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Patient 3</td>
<td>0.59%</td>
<td>0.52%</td>
</tr>
<tr>
<td>Patient 4</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Patient 5</td>
<td>4.47%</td>
<td>7.62%</td>
</tr>
</tbody>
</table>

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

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 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
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 not 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 $k_{\text{cat}}$ and $K_m$ values for phosphorylated E1 versus E1 plus phenylbutyrate and BDK). Phenylbutyrate not only increased the $k_{\text{cat}}$ (2–3-fold) but also boosted the sensitivity of the enzyme to BCKA by lowering their $K_m$ values (38–47%). Phenylbutyrate did not 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 $k_{\text{cat}}$ and $K_m$ values for phosphorylated E1 and E1 plus phenylbutyrate and BDK). Phenylbutyrate enhanced overall BCKDC activity as suggested by Paxton and Harris (12), BDK-catalyzed oxidation of all three BCKA substrates significantly. Phenylbutyrate, as determined by isothermal titration calorimetry (ITC), 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.
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
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 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/m²/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™ ODS2 column (250 mm x 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-1-14C-leucine for 4 h in the medium to stimulate the BCKDC activity. At the end of the incubation, the amount of 14CO₂ released from leucine decarboxylation is captured onto damped filter paper. Decarboxylation activity of BCKDC is expressed as pmol of CO₂ released/mg protein/hour and as percentage of normal activity. BDK activity was assayed by the incorporation of γ-32P-phosphate from [γ-32P]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 (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 KH₂PO₄, 1.2 mM MgCl₂, 25 mM NaHCO₃, 2 mM CaCl₂, 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 [1-14C]-Leu (specific activity around 200 dpm/nmol). The assay was carried out in a 20 ml scintillation vial and cells were gassed with a 95% O₂:5% CO₂ mixture for 20 s sealed with a rubber stopper and incubated in Dubnoff metabolic shaker at 37°C for

![Image of graph showing IC₅₀ value for inhibition of BDK by phenylbutyrate. The 100% control activity represents BDK activity in the absence of phenylbutyrate.](Human Molecular Genetics, 2011, Vol. 20, No. 4 637)
The reaction was stopped by addition of 3% perchloric acid and 
14CO2 trapped and quantified as described previously (38,39).
Branching-chain 13Cα-keto acid production from leucine (α-ketoisocaproyl) 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 microstatin, 33 mM 

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 MgCl2, 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 MgCl2, 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 (kapp) at different substrate concentrations for all of the above assays were determined from the absorption changes at the individual wavelength maximum. The kapp rate constants were fit using the following equation:

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

The phosphorylation of E1 was carried out in the phosphorylation reaction mix (30 mM HEPES, pH 7.4, 2 mM DTT, 1.5 mM MgCl2 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 dialed against 50 mM potassium phosphate buffer (pH 7.5) containing 50 mM potassium chloride and 2 mM MgCl2. 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.

ACKNOWLEDGEMENTS

The authors acknowledge the Texas Children’s Hospital General Clinical Research Center nursing and dietary staff. The authors thank clinical research staff of Mary Mullins, Susan Carter and Alyssa Tran. The authors would also acknowledge Terry Bertin and Dr Marzieh Taghavi for their technical assistance.

Conflict of Interest statement. None declared.
FUNDING

The work was supported in part by the Baylor College of Medicine General Clinical Research Center (RR0188 to B.L.); Mental Retardation and Developmental Disabilities Research Center (HD024064 to B.L.); the Child Health Research Center (HD041648 to B.L.), the Welch Foundation (I-1286 to D.T.C.) and the National Institute of Health [(DK54450 (to B.L.), RR019453, DK34738 and NS38642 (I-1286 to D.T.C.) and the National Institute of Health Medicine General Clinical Research Center (RR00188 to T.L.R.)]. The work was supported in part by the Baylor College of Medicine General Clinical Research Center (RR00188 to T.L.R.) and the National Institute of Health (DK081735); and by the National Urea Cycle Foundation Research Fellowship.

REFERENCES


