Phenylbutyrate increases pyruvate dehydrogenase complex activity in cells harboring a variety of defects

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Abstract

Objective: Deficiency of pyruvate dehydrogenase complex (PDHC) is the most common genetic disorder leading to lactic acidosis. PDHC deficiency is genetically heterogenous and most patients have defects in the X-linked E1-α gene but defects in the other components of the complex encoded by PDHB, PDHX, DLAT, DLD genes or in the regulatory enzyme encoded by PDP1 have also been found. Phenylbutyrate enhances PDHC enzymatic activity in vitro and in vivo by increasing the proportion of unphosphorylated enzyme through inhibition of pyruvate dehydrogenase kinases and thus, has potential for therapy of patients with PDHC deficiency. In the present study, we investigated response to phenylbutyrate of multiple cell lines harboring all known gene defects resulting in PDHC deficiency. In the present study, we investigated response to phenylbutyrate of multiple cell lines harboring all known gene defects resulting in PDHC deficiency. Methods: Fibroblasts of patients with PDHC deficiency were studied for their enzyme activity at baseline and following phenylbutyrate incubation. Drug responses were correlated with genotypes and protein levels by Western blotting. Results: Large deletions affecting PDHA1 that result in lack of detectable protein were unresponsive to phenylbutyrate, whereas increased PDHC activity was detected in most fibroblasts harboring PDHA1 missense mutations. In the prospect of a clinical trial, the results of this study may allow prediction of in vivo response in patients with PDHC deficiency harboring a wide spectrum of molecular defects.
**Introduction**

Deficiency of nuclear-encoded pyruvate dehydrogenase complex (PDHC) is one of the most common inborn errors of mitochondrial energy metabolism. PDHC catalyzes within mitochondria conversion of pyruvate to acetyl coenzyme A, a key regulatory step in the central pathway of energy production. This reaction is particularly important in brain which has obligatory requirement for aerobic glucose oxidation. Most patients with PDHC deficiency show progressive neurological degeneration and either persistent or episodic elevations of lactate in blood, cerebrospinal fluid (CSF), or both. Affected patients present with a graded spectrum of severity ranging from the most severe congenital brain malformations with overwhelming lactic acidemia and death in the neonatal period to development of Leigh syndrome or to a milder form with carbohydrate induced ataxia. PDHC deficiency may be due to mutations of PDHA1, encaging pyruvate dehydrogenase kinase (PDK) isoforms that inactivate the enzyme, whereas dephosphorylation by pyruvate dehydrogenase phosphatases (PDP1 and PDP2) restore PDHC activity. PDHC deficiency may be due to mutations in the genes PDHA1, PDHB, PDHX, DLAT, and DLD encoding for E1-α subunit by four pyruvate dehydrogenase kinase (PDK) isoforms that activate the enzyme, whereas dephosphorylation by pyruvate dehydrogenase phosphatase 1. The most common form is due to mutations of PDHA1 gene located on the short arm of the X chromosome. We have previously shown that phenylbutyrate increases residual activity of PDHC by increasing the proportion of unphosphorylated enzyme and has potential for therapy of PDHC deficiency and systemic lactic acidosis. In the present study, to correlate and has potential for therapy of PDHC deficiency and by increasing the proportion of unphosphorylated enzyme that phenylbutyrate increases residual activity of PDHC with the following primary monoclonal antibodies: anti-PDH-E1α (9H9AF5), anti-PDH-E1β (17A5E2H8), anti-PDH-E2 (15D3G9C11), anti-E3BP (13G2AE2BHS), and anti-PDH-E3 (EPR6635). The anti-PDH-E1α, anti-PDH-E1β, anti-PDH-E2, anti-E3BP mixed in a cocktail (4b110416), and the anti-PDH-E3 (4b113551) were purchased from Abcam (Cambridge, UK). The antibodies phosphodetect anti-PDH-E1α (pSer264; AP1062), phosphodetect anti-PDH-E1β (pSer203; AP1063), phosphodetect anti-PDH-E2 (pSer271; AP1064) were from Calbiochem (Darmstadt, Germany) and the anti-cytochrome c oxidase IV (COXIV; #4844) from Cell Signaling Technology Inc (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies (ECL rabbit IgG, HRP-linked whole antibody from donkey [GE Healthcare, Milan, Italy NA934] and ECL anti-mouse IgG, HRP-linked species-specific whole antibody from sheep [GE Healthcare, NA931]) were diluted in 5% dried skim milk and in 1% bovine serum albumin (BSA) in tris-buffered saline-Tween20. Protein bands were visualized with a chemiluminescence detection system (Thermo Fisher Scientific Inc., Rockford, IL, USA) and band intensities were quantified with Quantity One-4.6.7 Basic (1-D Analysis Software; Bio-Rad Laboratories Inc. Hercules, CA, USA).

For proteasome inhibition, cells were cultured in DMEM supplemented with 10% FBS and after the 5-day treatment with 1 mmol/L of phenylbutyrate, were incubated with 40 μmol/L MG132 (Selleckchem, Munich, Germany) or Dimethyl Sulfoxide (DMSO) for 6 h, washed once with cold phosphate-buffered saline (PBS), and scraped with RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.1% SDS) containing complete protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Samples were incubated for 20 min at 4°C, centrifuged at 16,000 g for 10 min and cell lysates were used for Western blot.

**Results**

We analyzed PDHC activity in untreated and phenylbutyrate-treated skin fibroblasts from patients harboring all with 10% fetal bovine serum (FBS) for 24 h or 5 days on mitochondrial extracts prepared from synchronized fibroblasts (cultured in DMEM and 1% FBS for 24 h), as previously described. PDHC enzyme activity was calculated as mmol min⁻¹ mg⁻¹ of protein and expressed as fold increase of activity in cells treated with phenylbutyrate over untreated cells (at least n = 2 for each treatment). Statistical significance was computed using the Student’s two-tailed test. P < 0.05 was considered statistically significant.

**Material and Methods**

PDHC activity was measured at baseline and after incubation with 1 mmol/L phenylbutyrate (Amonaps; Swedish Orphan International Lab, Stockholm, Sweden) in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 24 h or 5 days on mitochondrial extracts prepared from synchronized fibroblasts (cultured in DMEM and 1% FBS for 24 h), as previously described. PDHC enzyme activity was calculated as mmol min⁻¹ mg⁻¹ of protein and expressed as fold increase of activity in cells treated with phenylbutyrate over untreated cells (at least n = 2 for each treatment). Statistical significance was computed using the Student’s two-tailed test. P < 0.05 was considered statistically significant.

Fibroblasts were analyzed for expression of proteins of PDHC with the following primary monoclonal antibodies: anti-PDH-E1α (9H9AF5), anti-PDH-E1β (17A5E2H8), anti-PDH-E2 (15D3G9C11), anti-E3BP (13G2AE2BHS), and anti-PDH-E3 (EPR6635). The anti-PDH-E1α, anti-PDH-E1β, anti-PDH-E2, anti-E3BP mixed in a cocktail (4b110416), and the anti-PDH-E3 (4b113551) were purchased from Abcam (Cambridge, UK). The antibodies phosphodetect anti-PDH-E1α (pSer264; AP1062), phosphodetect anti-PDH-E1β (pSer203; AP1063), phosphodetect anti-PDH-E2 (pSer271; AP1064) were from Calbiochem (Darmstadt, Germany) and the anti-cytochrome c oxidase IV (COXIV; #4844) from Cell Signaling Technology Inc (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies (ECL rabbit IgG, HRP-linked whole antibody from donkey [GE Healthcare, Milan, Italy NA934] and ECL anti-mouse IgG, HRP-linked species-specific whole antibody from sheep [GE Healthcare, NA931]) were diluted in 5% dried skim milk and in 1% bovine serum albumin (BSA) in tris-buffered saline-Tween20. Protein bands were visualized with a chemiluminescence detection system (Thermo Fisher Scientific Inc., Rockford, IL, USA) and band intensities were quantified with Quantity One-4.6.7 Basic (1-D Analysis Software; Bio-Rad Laboratories Inc. Hercules, CA, USA).

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Table 1. PDHC-deficient fibroblasts.

| Cell line | Gender | Enzyme activity (% mean control) | Allele 1 nucleotide (protein)a | Allele 2 nucleotide (protein)a |
|-----------|--------|----------------------------------|-------------------------------|-------------------------------|
| **PDHA1 mutations** |       |                                   |                               |                               |
| 1b        | Male   | 62%                               | c.749C>T (p.Q259L)            | –                             |
| 2c        | Male   | 46%                               | c.787G>C (p.R263G)            | –                             |
| 3b        | Male   | 56%                               | c.832G>C (p.G278R)            | –                             |
| 4c        | Male   | 21%                               | c.1132G>C (p.R378C)           | –                             |
| 5b        | Male   | 45%                               | c.1132C>T (p.R378P)           | –                             |
| 6b        | Male   | 7%                                | c.1133G>A (p.R378G)           | –                             |
| 7b        | Male   | 18%                               | c.1133G>A (p.R378G)           | –                             |
| 8d        | Male   | 52%                               | c.483C>T (p.Y162C)            | –                             |
| 9b        | Male   | 7%                                | c.1050_1133dup84 (p.Q351_R378dup) | – |
| 10        | Female | 47%                               | c.302G>T (p.C72F)            | wt                            |
| 11b       | Female | 48%                               | c.302G>T (p.C72F)            | wt                            |
| 12b       | Female | 30%                               | c.355C>T (p.R90W)             | wt                            |
| 13b       | Female | 23%                               | c.379C>T (p.R98V)             | wt                            |
| 14b       | Female | 83%                               | c.787C>G (p.R263G)            | wt                            |
| 15        | Female | 13%                               | c.904C>T (p.R273C)            | wt                            |
| 16b       | Female | 21%                               | c.904C>T (p.R273C)            | wt                            |
| 17b       | Female | 19%                               | c.904C>T (p.R273C)            | wt                            |
| 18        | Female | 57%                               | c.924G>T (p.Q279H)            | wt                            |
| 19b       | Female | 35%                               | c.498C>T (p.R166C)            | wt                            |
| 20b       | Female | 8%                                | c.292-2A>G (p.E98_Q107del)    | wt                            |
| 21        | Female | 38%                               | c.1140_1162del (p.N381fsX43)  | wt                            |
| 22        | Female | 22%                               | c.934_940del (p.S312fsX12)    | wt                            |
| 23b       | Female | 19%                               | 2.14 Mb deletion             | wt                            |
| **PDHB mutations** | | 75% | c.301A>G (p.M101V) | c.301A>G (p.M101V) |
| 24b       | Male   | 70%                               | c.301A>G (p.M101V)            | c.301A>G (p.M101V)            |

(Continued)

Table 1. Continued.

| Cell line | Gender | Enzyme activity (% mean control) | Allele 1 nucleotide (protein)a | Allele 2 nucleotide (protein)a |
|-----------|--------|----------------------------------|-------------------------------|-------------------------------|
| 26b       | Female | 55%                               | c.301A>G (p.M101V)            | c.301A>G (p.M101V)            |
| 27        | Female | 26%                               | c.497A>G (p.Y137C)            | c.497A>G (p.Y137C)            |
| **PDHX mutations** | | 28b | Male | 25% | c.1426C>T (p.R476X) | c.1426C>T (p.R476X) |
| 29b       | Male   | 41%                               | c.1182+2T>C (p.I386fsX13)    | c.1182+2T>C (p.I386fsX13)    |
| 30b       | Male   | 35%                               | c.160+1G>A (p.G27X)          | c.965-1G>A (p.G27X)          |
| 31b       | Female | 25%                               | c.620delC (p.P207LfsX16)     | c.620delC (p.P207LfsX16)     |
| 32        | Male   | 14%                               | Exon 4 and 5 deletion        | Exon 4 and 5 deletion        |
| **DLAT mutations** | | 33b | Female | 13% | c.1624-1626del (p.V560del) | ND |
| **DLD mutations** | | 34b | Male | 28% | c.858dup (p.G287VfsX4) | c.1174G>A (p.V392M) |
| 35a       | Male   | 27%                               | c.105insA (p.Y35X)           | c.685G>T (p.G229C)           |
| **PDP1 mutations** | | 36f | Male | 33% | c.851_853del (p.L284del) | c.851_853del (p.L284del) |

wt, wild-type; ND, not determined.
aNucleotides are reported according to NCBI gene sequences (NM_000284.3 for PDHA1, NM_000925.3 for PDHB, NM_003477.2 for PDHX, NM_001931.4 for DLAT, NM_000108.3 for DLD, NM_001161781 for PDP1). The amino acid positions of the mutations are reported considering the mature NCBI protein sequences (NP_000275.1 for PDHA1 [361 amino acids], NP_000916.2 for PDHB [329 amino acids], NP_003468.2 for E3BP [448 amino acids], NP_000275.1 for PDHA1 [361 amino acids], NM_001931.4 for DLAT [561 amino acids], NP_000108.3 for DLD [474 amino acids], NP_001155253.1 for PDP1 [466 amino acids]) after signal peptide cleavage. Patients previously described by bImbard et al. 12, dBoichard et al. 10, eShaag et al. 28, and fMaj et al. 29.

Known molecular defects responsible for PDHC deficiency, that are, mutations in PDHA1, PDHB, PDHX, DLAT, DLD, and PDP1 genes (Table 1). As a control, enzyme activity was measured at baseline and after phenylbutyrate in fibroblasts from a control subject. Among 16 cell lines with PDHA1 missense mutations (Table 1), 11 cell lines showed a significant increase in enzyme activity following incubation with phenylbutyrate and had detectable E1-α protein by Western blotting (Fig. 1A and B). Fibroblasts from nine male patients harboring missense (p.P221L, p.R234G, p.G249R, p.R349C, p.R349H),
splicing (c.483C>T), or truncating (p.Q351_R378dup) mutations were studied (Table 1, black bars in Fig. 1A). Hemizygous cell lines from patients carrying missense mutations p.P221L, p.R234G, and p.G249R (cell lines 1, 2, and 3) showed elevated PDHC activities (62%, 46%, and 56% of control mean), detectable E1-α protein by Western blotting, and were all responsive to phenylbutyrate (Fig. 1A and B, and Table 1). Four cell lines not responsive to phenylbutyrate all carried PDHA1 missense mutations affecting the R349-α residue, had relatively lower levels of PDHC residual activity ranging from 7% to 45%, and showed very low-to-undetectable levels of protein E1-α by Western blotting, and were all responsive to phenylbutyrate (Fig. 1A and B, and Table 1). Four cell lines not responsive to phenylbutyrate all carried PDHA1 missense mutations affecting the R349-α residue, had relatively lower levels of PDHC residual activity ranging from 7% to 45%, and showed very low-to-undetectable levels of protein E1-α by Western blotting, and were all responsive to phenylbutyrate (Fig. 1A and B, and Table 1). Lack of increase in PDHC activity following phenylbutyrate incubation was also previously observed in another cell line (designated as Pt 3) from an unrelated patient also harboring a mutation affecting R349-α.7

It was previously shown that cells harboring the p.R349C mutation were also unresponsive to short-term incubation with dichloroacetate (DCA), a pyruvate analog which increases PDHC activity.9 In contrast, sustained DCA incubation reduced turnover of E1-α protein and increased PDHC activity.9 Therefore, we investigated longer incubation with phenylbutyrate in fibroblasts harboring mutations affecting R349-α (cell lines 4–7 and Pt 37) and found that 5-day drug incubation increased PDHC enzyme activity (Fig. 2A). Following 5-days of incubation with phenylbutyrate, we also observed an increase in the amount of E1-α protein compared to untreated cells (Fig. 2B). The ubiquitin-proteasome is the major route for degradation of cell proteins and thus, we investigated E1-α protein levels in fibroblast cell line 5, carrying the p.R349C mutation, by inhibiting proteasome activity with the MG132 proteasome inhibitor. Incubation with MG132 increased E1-α protein to levels similar to those observed in phenylbutyrate-treated cells but no further increase in E1-α was observed in cells cotreated with phenylbutyrate and MG132 (Fig. 2B). Taken together, these results may indicate that E1-α harboring the p.R349C mutation is degraded by the proteasome and phenylbutyrate increases resistance of the mutated protein to proteasome degradation.

The synonymous mutation c.483C>T (p.Y132Y) of cell line 8 causes aberrant splicing in 40% of total cDNA with exon 5 skipping predicted to result in a frameshift and premature termination after the addition of 10 aberrant amino acids (p.Arg141AlafsX11).10 Immunoblotting of these fibroblasts resulted in detectable E1-α protein band

**Figure 1.** (A) PDHC activity in fibroblasts with PDHA1 mutations. PDHC activity is shown as fold increase in baseline activity after incubation with phenylbutyrate. Bars indicate average ± standard error of the mean; *P < 0.05. PDHC activity was increased in four of the nine fibroblast cell lines from male patients shown as black bars and in 10 of the 14 fibroblast cell lines from female patients shown as white bars. (B) Western blotting of skin fibroblasts with PDHA1 mutations with a cocktail of antibodies recognizing E1-α (43 kDa), E1-β (39 kDa), E2 (69 kDa), and E3BP (54 kDa) proteins. Western blotting for cytochrome c oxidase (COX; 17 kDa) was performed as mitochondrial marker. The PDHA1 mutations of the corresponding patient cell lines are shown in Table 1. wt, wild-type control fibroblasts.
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of normal size expressed from the proportion of correctly
spliced mRNA (Fig. 1A). Phenylbutyrate likely increased
enzyme activity of normal E1-α enzyme expressed from
the correctly spliced mRNA. The hemizygous cell line 9
from male patient carrying the c.1050_1133dup84 resulted
in very low PDHC activity, in no detectable E1-α protein,
and phenylbutyrate failed to increase significantly PDHC
activity (Fig. 1A and B).

Fibroblasts from 14 female patients harboring missense
(p.C72F, p.R90W, p.R98W, p.R234G, p.R273C,
p.Q279H), splicing (c.498C>T), or truncating (p.E98_Q170del,
p.S312VfsX12, p.N381SfsX43, 2.15 Mb deletion)
mutations were studied (Table 1, white bars in Fig. 1A).
Cell lines with PDHA1 mutations from female patients
are expected to express both wild-type and mutant
alleles. Furthermore, skewed X-inactivation is found in
approximately one fourth of skin fibroblasts from PDHC-
deficient patients. Thus, PDHC activity in cells from
female patients results from both residual activity of the
enzyme encoded by the mutant allele and the activity of
the enzyme expressed from the wild-type allele. Phenylbu-
tyrate increases PDHC activity in wild-type cells
(Fig. 1A) and thus, it is not possible to determine
whether phenylbutyrate increases the activity of the
enzyme encoded by the wild-type or by the mutated allele
in cells from female patient. Nine fibroblast cell lines with
missense mutations resulted in variable PDHC activities
ranging from 13% to 83%, detectable levels of E1-α protein,
and were all responsive to phenylbutyrate incubation
(Fig. 1A and B). Three cell lines of fibroblasts
harboring splicing mutation or deletions (fibroblast cell
lines 20, 21, and 23) carrying the mutations c.292-
2A>G, c.1140_1162del, and a 2.14 Mb deletion, respec-
tively, showed detectable levels of E1-α protein and low
PDHC activity (8-38%) and were unresponsive to
phenylbutyrate (Fig. 1A and B). The absence or the
low levels of E1-α protein by Western blotting suggests
occurrence in these cell lines of skewed X-inactivation
favoring the mutant allele that could explain the lack of
response to phenylbutyrate.

Figure 2. (A) PDHC activity in fibroblasts with mutations affecting R349 residue of E1-α protein following shorter (1 day) and longer (5 days)
phenylbutyrate incubations. (B) Western blotting for E1-α (43 kDa), E1-β (39 kDa), and E2 (69 kDa) protein of cell line 5 harboring p.R349C
mutation incubated with either vehicle, phenylbutyrate, MG132, or a combination of phenylbutyrate and MG132. Cytochrome c oxidase (COX;
17 kDa) was used as mitochondrial control marker.
So far, 12 PDHB mutations have been reported and four mutations, including the p.M101V found in patients of North African descent,12 clustered in the same region.12–14 We investigated phenylbutyrate in four cell lines from patients harboring PDHB mutations (cell lines 24–27, Table 1) including the p.M101V mutation, and three of them showed a statistically significant increase in PDHC activity after phenylbutyrate incubation (Fig. 3A).

Three of these cell lines with PDHB mutations showed a reduction in E1-β protein by Western blotting, whereas the nonresponder cell line (cell 27) had E1-β levels similar to wild-type levels (Fig. 3B). In two of the three cell lines responding to phenylbutyrate in terms of increased enzyme activity (cell lines 24 and 25), an increase in E1-β protein was observed by Western blotting after phenylbutyrate incubation (Fig. 3B). In the three cell lines that responded to phenylbutyrate in terms of increased PDHC activity, a reduction in phosphorylated E1-α levels was observed, whereas cell line 27 that failed to respond to phenylbutyrate did not show a reduction in phosphorylated E1-α levels (Figs. 3B, S1).

The E3BP lacks catalytic activity and has a structural role in the formation of the enzyme complex binding E3 to E2. Mutations in PDHX gene encoding E3BP are the second most common cause of PDHC deficiency2,12 and the vast majority of the mutations are aberrant splicing or nonsense mutations.12 In this study, we investigated five cell lines harboring mutations in PDHX gene that all carried nonsense mutations or deletions and showed enzyme activity ranging from 14% to 41% (cell lines 28–32, Table 1, Fig. 3A and C). Two of the cell lines (31 and 32, Table 1) harboring a single nucleotide deletion leading to frameshift or an exon 4 and 5 deletion did not show detectable E3BP by Western blotting (Fig. 3C). All five
cell lines were found to be responsive to phenylbutyrate with an average 1.4-fold increase in enzyme activity (Fig. 3A). Phenylbutyrate incubation did not appear to affect E3BP protein levels determined by Western blotting and a reduction in phosphorylated E1α levels was observed in four of six cell lines (Figs. 3E, S1).

One cell line with DLAT mutation was available for analysis and a 1.3-fold increase enzyme activity was detected in this cell line after incubation with phenylbutyrate (cell line 33, Table 1 and Fig. 3A). Fibroblasts of cell line 33 had low enzyme activity and did not show detectable levels of E2 protein by Western blotting even after incubation with phenylbutyrate (Fig. 3D). Only one mutation was detected in this patient. It appears that mutations in the gene encoding the E2 are rare cause of PDHC deficiency.7,15-17 One patient previously reported had undetectable E2 immunoreactive protein,16 whereas two other patients were found to express reduced but detectable levels of E2 protein.17 The clinical presentation appeared to be more severe in the patient with undetectable E2 protein compared to the patients with detectable E2 that had relatively mild symptoms and episodic dystonia. DCA was previously shown to increase PDHC activity in fibroblasts of these patients with DLAT mutations.17 Phenylbutyrate was also found to correct the mitochondrial, locomotor, and behavioral phenotype of the noa<sup>631</sup> zebrafish model carrying a missense DLAT gene mutation.7 Thus, E2 defects resulting from DLAT mutations appear to be responsive to phenylbutyrate. A reduction in phosphorylated E1α levels was observed in this cell line with DLAT mutation following phenylbutyrate incubation (Figs. 1, 3B).

We also investigated phenylbutyrate in two cell lines harboring DLD mutations resulting in E3 deficiency and both showed an average 1.4-fold increase in baseline PDHC activity (cell lines 34 and 35, Table 1 and Fig. 3A). Both cell lines expressed the E3 protein, as shown by Western blotting with anti-E3 antibody (Fig. 3E). Clinical and biochemical presentation of this disorder is different from the common presentation of other PDHC defects.18,19 E3 is a common component of all three mitochondrial multienzyme complexes, and DLD mutations result in a disorder associated with a combination of features of branched chain a-ketoacid dehydrogenase (BCKDC), PDHC, and a-ketoglutarate dehydrogenase complex (KGDC) deficiencies. Phenylbutyrate inhibits the kinase of BCKDC (BDK) and PDKs<sup>7,20</sup> and thus, it is expected to result in improvements of both BCKDC and PDHC activities. Like cell lines with other defects, a reduction in phosphorylated E1α levels was observed in cells with DLD mutations (Figs. 3B, S1).

Finally, we investigated one cell line with PDP1 mutations that was found to respond to phenylbutyrate (cell line 36, Table 1; Fig. 3A). Phosphorylation of E1–α occurs at three serine residues (Ser264–α, site 1; Ser271–α, site 2; and Ser203–α, site 3). As expected based on the deficiency of the phosphatase activity, the amount of phosphorylated E1–α in this cell line is increased compared to control wild-type cell lines (Fig. 3F). Following phenylbutyrate incubation, phosphorylated E1–α was significantly reduced at all three phosphorylation sites (Fig. 3F) that is consistent with the mechanism of action of phenylbutyrate based on PDK inhibition.<sup>7</sup>

**Discussion**

Phenylbutyrate enhances PDHC activity in vitro and in vivo by increasing the proportion of unphosphorylated enzyme through inhibition of PDK. It also reduces lactic acid in a mouse model of systemic lactic acidosis, and rescues the phenotype of a zebrafish PDHC-deficient disease model.7 In the present study, we investigated the efficacy of phenylbutyrate in PDHC-deficient cells harboring a wide spectrum of genetic defects and we correlated genotypes with drug response. We found that phenylbutyrate increases PDHC activity in the majority of analyzed fibroblasts. Increased PDHC activity was detected in most of the fibroblasts with PDHA1 missense mutations. Large deletions or nonsense PDHA1 mutations resulting in no detectable protein by Western blotting were in general not responsive to phenylbutyrate. The gene encoding E1–α is located on the X chromosome, yet males and females are affected in approximately equal numbers due to the high proportion of heterozygous females manifesting the disease. All male patients with PDHA1 mutations have some degree of residual activity, whereas several female patients have null mutations resulting in complete deficiency of the enzyme subunit. It appears that complete PDHC deficiency is incompatible with normal development in hemizygous males but can be tolerated in heterozygous females due to the proportion of cells expressing the normal X chromosome. In cells from female patients, due to skewed pattern of X-inactivation favoring the normal X chromosome, enzyme activity might be higher, even falling within the normal range. In this context, the great majority of mRNA molecules arise from the normal gene. Phenylbutyrate increases PDHC activity in wild-type cells<sup>7</sup> and thus, it is not possible to determine in cells from female patients whether phenylbutyrate increases the activity of the enzyme encoded by the wild-type or by the mutated allele. Nevertheless, we cannot rule out that even an increase in PDHC activity in cells expressing the normal allele might be beneficial in patients with PDHC deficiency.

Cells harboring mutations affecting the R349–α residue of the E1–α protein were consistently found to be unresponsive to short-term incubation with phenylbutyrate.
but longer incubations resulted in increased activity. These results indicate that in addition to the direct effect on PDK, phenylbutyrate increases the stability of E1-α protein, at least in cells harboring missense mutations affecting the R349-α. This result is consistent with the previously recognized function of phenylbutyrate as chemical chaperone and with reduced E1-α protein turnover observed with sustained DCA incubation in addition to PDK inhibition by DCA.

We found that mutations affecting PDHB, PDHX, DLAT, and DLD genes were also responsive, thus potentially broadening therapeutic applications of phenylbutyrate to patients that harbor all known defects of PDHC deficiency. In these cell lines a reduction in phosphorylated E1-α protein levels was observed by Western blotting, thus suggesting that like cells with PDHA1 mutations, the increase in enzyme activity in cells harboring these defects is dependent, at least in part, on changes of phosphorylation status of the enzyme complex induced by phenylbutyrate.

Similar to phenylbutyrate, DCA also increases PDHC activity and is effective in reducing blood, CSF, and brain lactate. However, in a controlled trial in children with various forms of congenital lactic acidosis, chronic DCA administration did not result in improvements of neurologic problems and other clinical outcomes. Moreover, DCA has raised some concerns because it has been associated with hepatocellular and peripheral nerve toxicity, particularly in adults. In contrast, phenylbutyrate is a drug already approved for use in humans, its safety profile is well known, and thus it is attractive for therapy of patients with PDHC deficiency.

Enzyme activity measured in skin fibroblasts exhibit large variations among PDHC-deficient patients and does not correlate with the predicted severity of the mutation and cognitive outcomes, either in males or females. Nevertheless, enzyme activity appears to correlate with survival at least in males with PDHA1 mutations and thus, drug interventions aiming at increasing enzyme residual activity have potential for achieving clinically relevant results. However, it is unlikely that such drug interventions will reverse pre-existing severe brain damage, such as Leigh syndrome.

In summary, cells harboring PDHA1 gene mutations, that are the most common defects accounting for 80–90% of cases of PDHC deficiency with known mutations, respond to phenylbutyrate, especially if they carry missense mutations, have PDHC activity >10%, or exhibit detectable protein by Western blotting. Therefore, patients carrying these defects are candidates for such drug intervention. Cell lines from patients carrying other less common molecular defects also appear to be responsive to phenylbutyrate.

In conclusion, the results of the present study may help to predict whether a patient with PDHC deficiency will be responsive to phenylbutyrate therapy based on the affected gene, type, and location of the mutation.

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Authors Contributions
R. Ferriero performed all experiments included in the study. A. Boutron, M. Brivet, D. Kerr, E. Morava, R. J. Rodenburg, L. Bonafé, M. R. Baumgartner, Y. Anikster, and N. E. Braverman provided fibroblast cell lines from PDHC-deficient patients with molecular diagnoses. N. Brunetti-Pierri designed and supervised the study.

Conflict of Interest
None declared.

References
1. Robinson BH. Lactic acidemia and mitochondrial disease. Mol Genet Metab 2006;89:3–13.
2. Patel KP, O’Brien TW, Subramony SH, et al. The spectrum of pyruvate dehydrogenase complex deficiency: clinical, biochemical and genetic features in 371 patients. Mol Genet Metab 2012;105:34–43.
3. DeBrosse SD, Okajima K, Zhang S, et al. Spectrum of neurological and survival outcomes in pyruvate dehydrogenase complex (PDC) deficiency: lack of correlation with genotype. Mol Genet Metab 2012;107:394–402.
4. Korotchkina LG, Patel MS. Mutagenesis studies of the phosphorylation sites of recombinant human pyruvate dehydrogenase. Site-specific regulation. J Biol Chem 1995;270:14297–14304.
5. Huang B, Gudi R, Wu P, et al. Isoenzymes of pyruvate dehydrogenase phosphatase. DNA-derived amino acid sequences, expression, and regulation. J Biol Chem 1998;273:17680–17688.
6. Brown RM, Brown GK. X chromosome inactivation and the diagnosis of X linked disease in females. J Med Genet 1993;30:177–184.
7. Ferriero R, Manco G, Lamantea E, et al. Phenylbutyrate therapy for pyruvate dehydrogenase complex deficiency and lactic acidosis. Sci Transl Med 2013;5:175ra31.
8. DeVivo DC, Haymond MW, Obert KA, et al. Defective activation of the pyruvate dehydrogenase complex in...
subacute necrotizing encephalomyelopathy (Leigh disease). Ann Neurol 1979;6:483–494.
9. Fouque F, Brivet M, Boutron A, et al. Differential effect of DCA treatment on the pyruvate dehydrogenase complex in patients with severe PDHC deficiency. Pediatr Res 2003;53:793–799.
10. Boichard A, Venet L, Naas T, et al. Two silent substitutions in the PDHA1 gene cause exon 5 skipping by disruption of a putative exonic splicing enhancer. Mol Genet Metab 2008;93:323–330.
11. Otero LJ, Brown RM, Brown GK. Arginine 302 mutations in the pyruvate dehydrogenase E1alpha subunit gene: identification of further patients and in vitro demonstration of pathogenicity. Hum Mutat 1998;12:114–121.
12. Imbard A, Boutron A, Vequaud C, et al. Molecular characterization of 82 patients with pyruvate dehydrogenase complex deficiency. Structural implications of novel amino acid substitutions in E1 protein. Mol Genet Metab 2011;104:507–516.
13. Okajima K, Korotchikina LG, Prasad C, et al. Mutations of the E1beta subunit gene (PDHB) in four families with pyruvate dehydrogenase deficiency. Mol Genet Metab 2008;93:371–380.
14. Quintana E, Mayr JA, Garcia Silva MT, et al. PDH E1beta deficiency with novel mutations in two patients with Leigh syndrome. J Inherit Metab Dis 2009;32 (Suppl. 1):S339–S343.
15. McWilliam CA, Ridout CK, Brown RM, et al. Pyruvate dehydrogenase E2 deficiency: a potentially treatable cause of episodic dystonia. Eur J Paediatr Neurol 2010;14:349–353.
16. Robinson BH, MacKay N, Petrao-Benedict R, et al. Defects in the E2 lipoyl transacetylase and the X-lipoyl containing component of the pyruvate dehydrogenase complex in patients with lactic acidemia. J Clin Invest 1990;85:1821–1824.
17. Head RA, Brown RM, Zolkiipli Z, et al. Clinical and genetic spectrum of pyruvate dehydrogenase deficiency: dihydrolipoamide acetyltransferase (E2) deficiency. Ann Neurol 2005;58:234–241.
18. Odievre MH, Chretien D, Munnich A, et al. A novel mutation in the dihydrolipoamide dehydrogenase E3 subunit gene (DLD) resulting in an atypical form of alpha-ketoglutarate dehydrogenase deficiency. Hum Mutat 2005;25:323–324.
19. Cameron JM, Levandovskiy V, Mackay N, et al. Novel mutations in dihydrolipoamide dehydrogenase deficiency in two cousins with borderline-normal PDH complex activity. Am J Med Genet A 2006;140:1542–1552.
20. Brunetti-Pierri N, Lanpher B, Erez A, et al. Phenylbutyrate therapy for maple syrup urine disease. Hum Mol Genet 2011;20:631–640.
21. Cao SS, Zimmermann EM, Chuang BM, et al. The unfolded protein response and chemical chaperones reduce protein misfolding and colitis in mice. Gastroenterology 2013;144:e6.
22. Morten KJ, Caky M, Matthews PM. Stabilization of the pyruvate dehydrogenase E1alpha subunit by dichloroacetate. Neurology 1998;51:1331–1335.
23. Morten KJ, Beattie P, Brown GK, Matthews PM. Dichloroacetate stabilizes the mutant E1alpha subunit in pyruvate dehydrogenase deficiency. Neurology 1999;53:612–616.
24. Stacpoole PW, Gilbert LR, Neiberger RE, et al. Evaluation of long-term treatment of children with congenital lactic acidosis with dichloroacetate. Pediatrics 2008;121:e1223–e1228.
25. Stacpoole PW, Kerr DS, Barnes C, et al. Controlled clinical trial of dichloroacetate for treatment of congenital lactic acidosis in children. Pediatrics 2006;117:1519–1531.
26. Kaufmann P, Engelstad K, Wei Y, et al. Dichloroacetate causes toxic neuropathy in MELAS: a randomized, controlled clinical trial. Neurology 2006;66:324–330.
27. Tuchman M, Lee B, Lichter-Konecki U, et al. Cross-sectional multicenter study of patients with urea cycle disorders in the United States. Mol Genet Metab 2008;94:397–402.
28. Shaag A, Saada A, Berger I, et al. Molecular basis of lipoamide dehydrogenase deficiency in Ashkenazi Jews. Am J Med Genet 1999;82:177–182.
29. Maj MC, MacKay N, Levandovskiy V, et al. Pyruvate dehydrogenase phosphatase deficiency: identification of the first mutation in two brothers and restoration of activity by protein complementation. J Clin Endocrinol Metab 2005;90:4101–4107.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Average intensities of Western blotting bands of phosphorylated E1-α normalized for total E1-α from two independent experiments of fibroblasts with PDHB, PDHX, DLAT, and DLD mutations (Table 1) incubated with phenylbutyrate or vehicle. *P < 0.05.