Genetic Basis for Correction of Very-Long-Chain Acyl–Coenzyme A Dehydrogenase Deficiency by Bezafibrate in Patient Fibroblasts: Toward a Genotype-Based Therapy

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Very-long-chain acyl–coenzyme A dehydrogenase (VLCAD) deficiency is an inborn mitochondrial fatty-acid β-oxidation (FAO) defect associated with a broad mutational spectrum, with phenotypes ranging from fatal cardiopathy in infancy to adolescent-onset myopathy, and for which there is no established treatment. Recent data suggest that bezafibrate could improve the FAO capacities in β-oxidation–deficient cells, by enhancing the residual level of mutant enzyme activity via gene-expression stimulation. Since VLCAD-deficient patients frequently harbor missense mutations with unpredictable effects on enzyme activity, we investigated the response to bezafibrate as a function of genotype in 33 VLCAD-deficient fibroblasts representing 45 different mutations. Treatment with bezafibrate (400 μM for 48 h) resulted in a marked increase in FAO capacities, often leading to restoration of normal values, for 21 genotypes that mainly corresponded to patients with the myopathic phenotype. In contrast, bezafibrate induced no changes in FAO for 11 genotypes corresponding to severe neonatal or infantile phenotypes. This pattern of response was not due to differential inductions of VLCAD messenger RNA, as shown by quantitative real-time polymerase chain reaction, but reflected variable increases in measured VLCAD residual enzyme activity in response to bezafibrate. Genotype cross-analysis allowed the identification of alleles carrying missense mutations, which could account for these different pharmacological profiles and, on this basis, led to the characterization of 9 mild and 11 severe missense mutations. Altogether, the responses to bezafibrate reflected the severity of the metabolic blockage in various genotypes, which appeared to be correlated with the phenotype, thus providing a new approach for analysis of genetic heterogeneity. Finally, this study emphasizes the potential of bezafibrate, a widely prescribed hypolipidemic drug, for the correction of VLCAD deficiency and exemplifies the integration of molecular information in a therapeutic strategy.

Very-long-chain acyl–coenzyme A (CoA) dehydrogenase (VLCAD) deficiency (MIM 609575) is an autosomal recessive genetic disorder first identified in 1993[1] and now considered one of the more common mitochondrial β-oxidation disorders,[2] with an estimated incidence of 1 in 30,000 to 1 in 120,000 births.[3,4] The associated disease is clinically heterogeneous in terms of age at onset, severity, and affected tissues, but three main phenotypes have been described.[5] The most severe form of VLCAD deficiency presents with neonatal cardiomyopathy and hepatic failure and is generally fatal in the 1st year of life. An infantile presentation, mainly characterized by episodes of hypoketotic hypoglycemia in early childhood, has also been described. Finally, the most common phenotype is an adolescent- or adult-onset myopathy with myalgia, cramps, and exercise intolerance, with associated rhabdomyolysis.[2] This phenotype complexity suggests that different therapeutic approaches will be necessary for appropriate management of this disease; however, there is yet no established therapy for VLCAD deficiency. Current management is largely supportive and is based on the administration of a high-carbohydrate, low long-chain fat diet supplemented with medium-chain triglycerides. Because of the severity of the symptoms and because life-threatening manifestations can occur even in otherwise mild phenotypes,[6] newborn screening of VLCAD deficiency has been implemented in most U.S. states, Australia, and some European countries.[4] Characterization of abnormal plasma acylcarnitines by tandem mass spectrometry allows the detection of suspected deficiency in newborns, and definitive diagnosis is based on fatty-acid oxidation (FAO) studies in fibroblasts or lymphocytes and/or sequence analysis of the VLCAD gene.[3,4] The majority of patients identified by neonatal screening are asymptomatic at diagnosis and are treated prospectively with preventive dietary measures, contributing to a decrease in

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disease mortality. However, these patients appear to frequently develop muscular manifestations of the disease, but the likelihood of illness and the long-term outcome are so far not widely documented.

The diversity of disease presentations is clearly associated with a high genetic heterogeneity; however, the genotype-phenotype correlations are not completely understood. More than 80 mutations, spread throughout the 20 exons of the VLCAD gene (17p13), have been reported, and none of them are prevalent.[2] The most-severe disease presentations are often associated with null-type mutations (i.e., large deletions and truncating, nonsense, or splice mutations), which prevent the formation of any functional protein and can account for the severity of the associated phenotypes.[2,5] However, these null mutations represent only one-third of disease-associated sequence variations, and the majority of patients harbor missense mutations with potentially variable effects, and these can be found in all three phenotypes.[5,7] With the exception of a few mutations that target the catalytic sites, predictions of the effects of missense mutations are often uncertain and require experimental studies. Functional analysis of mutations by cDNA expression studies have been performed in a limited number of cases but could not practically be extended to the spectrum of mutations already known in this disease. Biochemical studies of patient cells might provide an alternative for analysis of the disease variability. In many cases, however, measurements of FAO capacities or residual VLCAD enzyme activity, as well as studies of acylcarnitine profiles in the patient cells, do not reliably discriminate among the various forms of the disease[8] and do not clearly correlate with the various genotypes.[5] Thus, despite marked advances in diagnosis and molecular characterization of the disease, the relations among the genotype variability, the severity of the metabolic block, and the different possible phenotypes are poorly understood in many VLCAD-deficient patients. This obviously limits molecular-based predictions regarding the evolution of the disease or the optimal case management, especially in the case of asymptomatic newborns, in which the molecular diagnosis remains, for these reasons, of limited prognostic value.

In several genetic disorders, functional analysis of mutations has proven to be essential not only for diagnosis but also for the design of adapted therapeutic approaches. For example, the pharmacological rescue of mutant proteins that are misfolded, that exhibit abnormal cell trafficking, or that are constitutively activated is now considered a possible option for the correction of various genetic diseases, including cystic fibrosis, Pompe disease, or neonatal diabetes, illustrating that a combined pharmacological and genetic approach can offer the prospect of targeting genotype-specific therapies.[9–12] In a recent study, we established that pharmacological enhancement of a deficient enzyme could be achieved in cells carrying mild mutations of the *CPT2* (carnitine palmitoyl transferase 2) gene, and this could lead to the normalization of FAO capacities.[13,14] This was achieved through cell exposure to bezafibrate, a drug widely used for its hypolipidemic action[15] and acting as an agonist of the peroxisomal proliferator-activated receptors (PPARs). Upon pharmacological activation, PPARs trigger an up-regulation of *CPT2* gene expression, which results in an increase in CPT2 residual enzyme activity and thereby correction of FAO flux in treated cells. This approach can conceivably be extended to other FAO defects, since the PPAR signaling pathway controls many different enzymes in the β-oxidation pathway. In keeping with this hypothesis, a pilot study established a beneficial effect of bezafibrate in a small series of VLCAD-deficient fibroblasts.[16] However, as for the correction of other genetic diseases on the basis of a similar strategy, it can be anticipated that the drug effects will closely depend on the ability to produce a partially active mutant protein. Since most VLCAD-deficient patients harbor mutations with variable and unpredictable effects on the enzyme properties,[7] the potential of bezafibrate for correction of VLCAD deficiency cannot be anticipated from the nature of these mutations, and it appeared essential to analyze the responsiveness to the drug in relation to the genotype variability. On this basis, we investigated the effects of bezafibrate on FAO capacities in a large and genetically heterogeneous panel of VLCAD-deficient cells and focused in particular on the study of genotypes carrying missense mutations.

We hypothesized that this pharmacological approach could provide a way to compare the functional consequences of various genotypes, or of various mutations, on the cell metabolic capacities. The other objective of this study was to characterize the nature and the proportion of VLCAD-deficient genotypes on which bezafibrate could have beneficial effects—that is, could improve or correct the defect in FAO capacity—in a relatively large sample of patient cells.

Subjects and Methods

Patients and Control Cells

VLCAD-deficient human skin fibroblasts were obtained with consent from different reference centers for the diagnosis of inborn metabolic diseases. All cell lines had been previously genotyped: their genotypes are given with references in table 1 and are positioned along the VLCAD gene in figure 1. The case histories of some of the patients included in this study have been published previously.[5,16–27] An indication of the phenotype at the time of diagnosis is included in table 1. The neonatal (N) phenotype presented with cardiomyopathy, often in association with liver failure, possibly resulting in death within the 1st year of life. The infantile (I) form was characterized by liver failure with hypoketotic hypoglycemia, without heart or muscle involvement. Finally, the myopathic (M) phenotype included patients with metabolic myopathy (i.e., myalgia, exercise intolerance, and rhabdomyolysis) generally of adolescent or adult onset, except for genotypes 5, 12, 18, and 25, in which muscular manifestations developed earlier (before age 8 years). Control fibroblast cell lines were from healthy individuals.

Table 1. Genotypes of the VLCAD-Deficient Patients

	Allele 1			Allele 2				
Genotype	Nucleotide Change[a]	Exon(s)	Amino Acid Change[b]	Nucleotide Change[a]	Exon(s)	Amino Acid Change[b]	Phenotype[c]	Reference(s)[d]
1	c.37C→T	1	Q13X	c.856A→G	9	R286G	N	…
2	c.102delC	2	G34fsX60	c.102delC	2	G34fsX60	N	Andresen et al.[5]
3	c.194C→T+c.739A→C	3 and 8	P65L+K247Q	c.194C→T+c.739A→C	3 and 8	P65L+K247Q	N	Watanabe et al.[27]
4	c.265C→T	4	P89S	c.1606_1609del	17	A536fsX550	I*	Takusa et al.[25]
5	c.272C→A	4	P91Q	c.577G→A	7	G193R	M*	Engbers et al.[19]
6	c.364A→G	6	N122D	c.364A→G	6	N122D	I	Djouadi et al.[16]
7	c.388_391del	6	E130fsX216	c.1144A→C	11	K382Q (7)	N	Aoyama et al.[18]
8	c.442A→G	6	S148G	c.1531C→T	15	R511W	M	…
9	c.520G→A	7	V174M	c.520G→A	7	V174M	M	Djouadi et al.[16]
10	c.553G→A	7	G185S (1)	IVS8-2A→C	9	N252_H293del (3)	N	Andresen et al.,[5] Pons et al.[22]
11	c.553G→A	7	G185S (1)	c.878G→A	10	G294E	I*	Andresen et al.[5]
12	c.664G→A	8	G222R (2)	c.664G→A	8	G222R (2)	M*	…
13	c.664G→A	8	G222R (2)	c.1512G→T	15	E504D	M	…
14	c.685C→T	8	R229X	c.1837C→T	20	R613W	N	…
15	IVS8-2A→C	9	N252_H293del (3)	c.1322G→A	13	G441D (9)	N	Andresen et al.,[17] Pons et al.[22]
16	c.779C→T	9	T260M	c.1918_1921del	20	A640fsX679	N	Andresen et al.[17]
17	c.790A→G	9	K264E (4)	c.790A→G	9	K264E (4)	M	Takusa et al.[25]
18	c.790A→G	9	K264E (4)	c.997insT	10	A333fsX358 (6)	M*	Takusa et al.[25]
19	c.790A→G	9	K264E (4)	c.1309A→G	13	M437V	M	Takusa et al.[25]
20	c.848T→C	9	V283A (5)	c.848T→C	9	V283A (5)	M	Djouadi et al.[16]
21	c.848T→C	9	V283A (5)	c.1322G→A	13	G441D (9)	M	Andresen et al.[5,17]
22	c.848T→C	9	V283A (5)	c.1384G→A	14	E462K	M	Andresen et al.[5]
23	c.896C→T	10	K299M	c.1505T→A	15	L502Q	M	Tong et al.[26]
24	c.910G→A	10	A304T	c.1316G→A	12	G439D	M	…
25	c.997insT	10	A333fsX358 (6)	c.1144A→C	11	K382Q (7)	M*	…
26	c.1097G→A	11	R366H	c.1358G→T	14	R453X	M	Andresen et al.[5]
27	c.1144A→C	11	K382Q (7)	c.1339G→A	14	G447R	M	Souri et al.[24]
28	c.1213G→C	12	D405H	c.1349G→A	14	R450H (10)	M	Smelt et al.[23]
29	c.1246G→A	12	A416T (8)	c.1349G→A	14	R450H (10)	M	Fukao et al.[20]
30	c.1246G→A	12	A416T (8)	c.1798delA	19	K600fsX679	M	Takusa et al.[25]
31	c.1358G→A	14	R453Q	c.1358G→A	14	R453Q	N	Andresen et al.[5]
32	c.1367G→A	14	R456H	c.1844G→A	20	R615Q	M	Andresen et al.,[5] Mathur et al.[21]
33	c.1405C→T	14	R469W	c.1405C→T	14	R469W	N	Andresen et al.[5]

[a] The numbering of nucleotides starts at the first adenine of the translation initiation codon.

[b] The numbering of amino acids starts at the first methionine encoded by the translation initiation codon. Numbers 1–9 (in parentheses) indicate the genotypes that have this mutation in common.

[c] Phenotype at the time of diagnosis. I = infantile; M = muscular; N = neonatal. An asterisk (*) indicates moderate infantile or severe muscular forms.

[d] References describing the genotype or only one of the two mutations.

Cell Culture

Fibroblasts from normal or VLCAD-deficient cells were grown in Ham's F10 media (Invitrogen) with glutamine, 12% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin under standard conditions, as described elsewhere.[16] For treatment, cells were incubated in fresh media containing 400 μM bezafibrate (Sigma) or the equivalent amount of dimethyl sulfoxide (vehicle) for 48 h.

FAO

FAO flux was determined by quantifying the production of ^3H_2O from (9,10-^{3}H) palmitate (Perkin Elmer), as described elsewhere.[28] Palmitate bound to fatty-acid–free albumin was used at the final concentration of 125 mM (60 Ci/mmol). The oxidation rates were expressed as nanomoles of ^{3}H fatty acid oxidized per hour per milligram of cell protein (nmol ^{3}H FA/h/mg protein).

Enzyme Activity

VLCAD activity was measured as 2,3-unsaturated-palmitoyl-CoA and β-hydroxypalmitoyl-CoA formed from palmitoyl-CoA by high-performance liquid chromatography, as described elsewhere.[29] It was expressed as nanomoles of 2,3-unsaturated-palmitoyl-CoA formed per minute per milligram of cell protein (nmol/min/mg protein).

Real-Time Quantitative PCR

Total RNA from controls or VLCAD-deficient patients was isolated using Trizol reagent (Invitrogen) and was transcribed into cDNA. VLCAD and β-actin transcripts were amplified with primers described elsewhere[16] and were measured by real-time quantitative PCR by use of the SYBR Green technology kit (Abgene) and the LightCycler instrument (Roche). The results are expressed in arbitrary units as fold changes in VLCAD mRNA levels, normalized

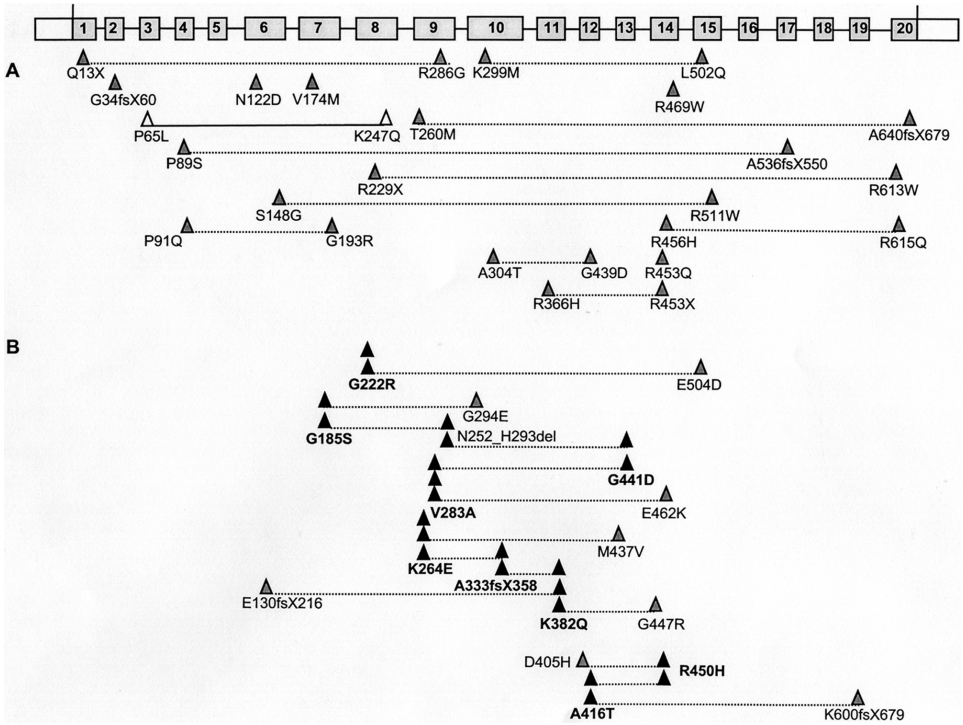

Figure 1. Schematic representation of the different genotypes along the VLCAD gene. Homozygous genotypes are indicated by a single triangle, and heterozygous genotypes are indicated by two triangles linked by a dashed line. One homozygous double point mutation (P65L+K247Q) is indicated by triangles linked by a continuous line. *A,* Genotypes with no mutation in common. *B,* Genotypes with mutations in common. The common mutations are indicated by dark triangles.

to the amount of β-actin, in treated cells relative to untreated ones.

Protein Sequence Analysis

Human and rat VLCAD (GenBank accession numbers P49748 and P45953, respectively), human, pig, and rat medium-chain acyl-CoA dehydrogenase (MCAD [GenBank accession numbers P11310, P41367, and P08503, respectively]), and human and rat short-chain acyl-CoA dehydrogenase (SCAD [GenBank accession numbers P16219 and P15651, respectively]) protein sequences were aligned using the online Multi-Align Tool, to identify conserved residues.

Predictive Three-Dimensional Model

The crystal structure of the VLCAD protein has not yet been elucidated, and the proposed three-dimensional model of VLCAD is derived from the established MCAD model.[30] Amino acid sequence homology between VLCAD and MCAD is 33% for residues 77–480 of VLCAD, indicating that the N-terminal domains of both enzymes are very similar. The structure of this N-terminal VLCAD domain (residues K79–K480) was modeled with the comparative protein structure modeling program, MODELLER,[31] with use of the structure of MCAD in complex with octanoyl-CoA (Protein Data Bank code 3MDE) as a template. The structure of the 180-residue C-terminal domain of VLCAD anchored to the mitochondrial membrane, which is absent from MCAD, is presently unknown.

Results

Effects of Bezafibrate on FAO Capacities in VLCAD-Deficient Fibroblasts: Can the Response to Bezafibrate Reveal the Residual FAO Capacities of a Given Genotype?

Determinations of basal palmitate oxidation rates in untreated cultured fibroblasts (fig. 2) revealed that most patient cell lines exhibited a significant deficiency in long-chain FAO capacities, compared with the control values (4.8 ± 1.0 nmol ^{3}H FA/h/mg protein; $n = 5$). Indeed, only 4 of the 33 cell lines (genotypes 13, 17, 32, and 21) exhibited FAO rates (3.7–4.5 nmol ^{3}H FA/h/mg protein) close to the control range. In the other 29 cell lines, the FAO rates varied from an upper value of 3 nmol ^{3}H FA/h/mg protein (genotype 19, −38% relative to mean control value) to barely detectable levels (genotypes 3, 10, and 14, complete FAO deficiency). Overall, the FAO-deficient genotypes included 18 cell lines with residual FAO capacities of 0–1 nmol ^{3}H FA/h/mg protein (FAO deficiency >80% compared with control), 9 cell lines with FAO rates of 1–2 nmol ^{3}H FA/h/mg protein (relative FAO deficiency from −58% to −80%), and 2 cell lines with a residual FAO flux of 2–3 nmol ^{3}H FA/h/mg protein (FAO deficiency from −58% to −38%), in the absence of treatment with bezafibrate.

Addition of 400 μM bezafibrate for 48 h to the cultured

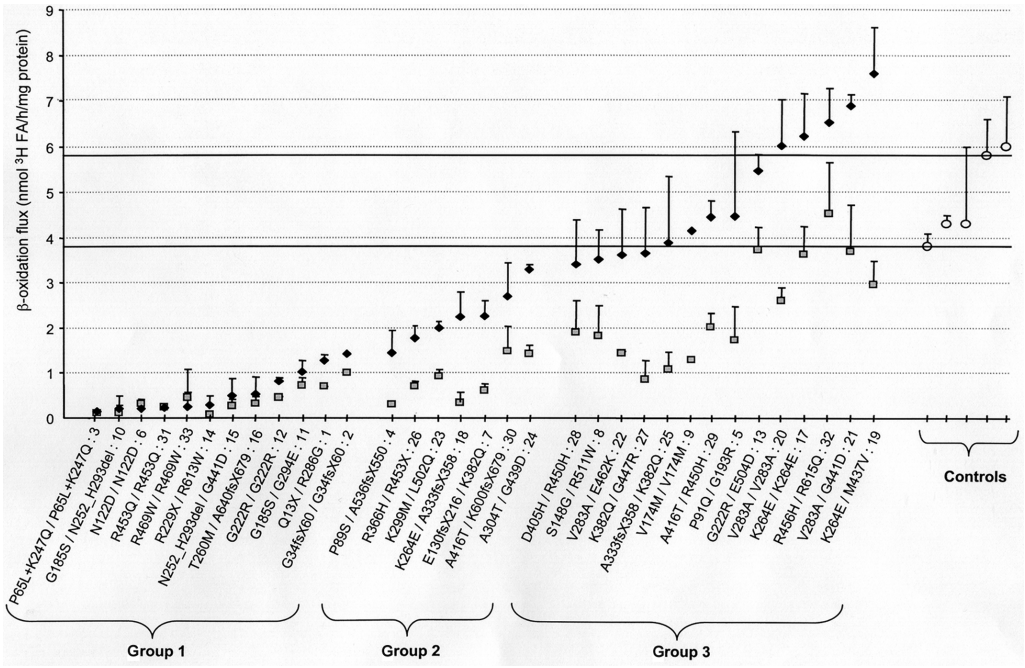

Figure 2. β-oxidation flux measured in fibroblasts of control and VLCAD-deficient patients before (gray square) and after (blackened rhombus) treatment with bezafibrate (400 μM for 48 h). In each experiment, the assays were performed in triplicate. The results are means $\pm$ SDs from at least three different experiments. For control values, five different healthy individuals were considered. The control ranges (untreated cells) represented by the horizontal lines are the mean + 1 SD and the mean − 1 SD of the five controls. Each genotype is numbered as in table 1.

fibroblasts induced major changes in the FAO capacities in about two-thirds (21 of 33) of the cell lines considered. These effects of bezafibrate on FAO are depicted in figure 2, in which the various genotypes were ranked according to the level of palmitate oxidation reached after cell exposure to the drug, revealing the maximal residual FAO capacities of each cell line. Analysis of the FAO data clearly identified three groups. In the first group (n = 12), bezafibrate induced no significant changes in FAO capacities, which remained extremely low compared with normal (<1.5 nmol ^{3}H FA/h/mg protein). The second group included seven cell lines in which bezafibrate induced a significant (1.5- to 3.5-fold) increase in palmitate oxidation but did not fully restore FAO capacities. Indeed, bezafibrate-treated group 2 cells exhibited palmitate oxidation rates varying from 1.5 to 3.3 nmol ^{3}H FA/h/mg protein compared with a control range of 3.8–5.8 nmol ^{3}H FA/h/mg protein (untreated cells). Finally, the third group comprised 14 VLCAD-deficient cells, in which bezafibrate induced full restoration of palmitate oxidation. Of these 14 cell lines, 10 were initially deficient compared with control fibroblasts. The results obtained in this group showed that treatment with bezafibrate could, at least in some cases, correct major FAO deficiencies such as those found in untreated cells of genotypes 22, 27, 25, or 9 (from −75% to −65% relative to control).

Fibroblast Response to Bezafibrate in Relation to the Disease Phenotype

Analysis of FAO results in relation to patient phenotypes showed that 11 (>90%) of the 12 group 1 cell lines that did not respond to bezafibrate came from patients with a severe (neonatal or infantile) presentation of the disease. The only patient with a myopathic phenotype found in group 1 (genotype 12) presented severe muscular manifestations early in life, corresponding to an aggravated myopathic phenotype. On the other hand, 19 (>90%) of 21 cell lines in which bezafibrate induced a significant increase in FAO (groups 2 and 3) originated from patients with the myopathic phenotype.

Changes in VLCAD mRNA Abundance in Cells Treated with Bezafibrate

The results from real-time quantitative PCR studies reported in figure 3 showed that exposure to bezafibrate generally induced significant increases in VLCAD mRNA levels in the various cell lines. Interestingly, the changes in VLCAD mRNA triggered by bezafibrate were equivalent in the group 1 (from 1.2-fold to 2.1-fold), group 2 (from 1.2-fold to 2.5-fold), and group 3 (from 1.3-fold to 2.3-fold) genotypes. Furthermore, these variations were quite comparable with those measured in control fibroblasts (n = 4), in which VLCAD mRNA increased by a factor of

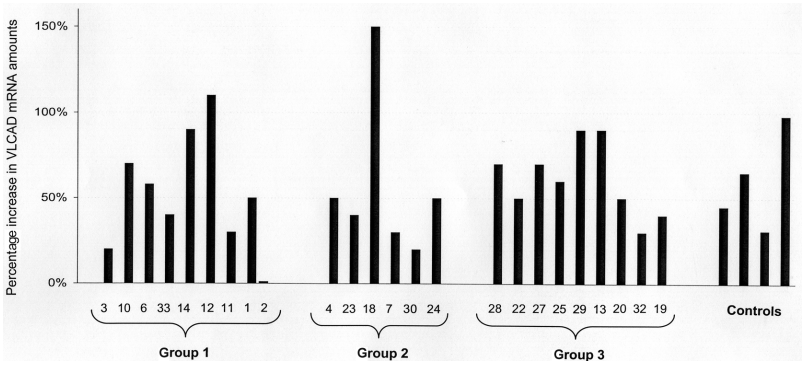

Figure 3. Induction of VLCAD mRNA by bezafibrate. The results are expressed as the percentage increase relative to vehicle-treated cells.

1.3–2 after treatment with bezafibrate. Only one cell line (genotype 2, group 1) exhibited extremely low basal VLCAD mRNA levels that did not vary in response to bezafibrate, consistent with the fact that the corresponding mutations cause mRNA degradation by the nonsense-mediated mRNA decay system.[7]

Induction of VLCAD Activity in Drug-Treated Cells

Enzyme activity determinations showed that untreated VLCAD-deficient fibroblasts from group 1 exhibited no or barely detectable residual VLCAD activity (fig. 4). The basal levels observed in groups 2 and 3, which ranged from 0.4 to 3.3 nmol/min/mg protein, were slightly higher than those in group 1 but still remain markedly deficient compared with the values found in control fibroblasts (11 ± 1.9 nmol/min/mg protein; $n = 3$). Figure 4 shows that the stimulation of the residual enzyme activity of VLCAD by bezafibrate in group 3 ranged from 2.2- to 4.8-fold compared with the values in untreated cells. The levels of VLCAD enzyme activities in the bezafibrate-treated group 3 fibroblasts were close to normal in two cases (7.1 and 8.9 nmol/min/mg protein in genotypes 20 and 17, respectively) but generally remained deficient in all the other cell lines, despite the induction triggered by bezafibrate.

Altogether, VLCAD mRNA levels were induced in the large majority of patient fibroblasts treated with bezafibrate, irrespective of the nature of the point mutation(s). In contrast, the response to drug treatment in terms of changes in FAO capacities markedly differed among the various genotypes. In the genotypes classified as group 3, drug-induced increases in VLCAD mRNA clearly led to a stimulation of residual enzyme activity that accounted for the restoration of palmitate oxidation rates. In contrast, in genotypes that were ranked in group 1, increases in the steady-state level of VLCAD mRNA did not translate into a stimulation of FAO capacities because of the absence of changes in VLCAD residual activity, which remained extremely low despite treatment with bezafibrate. Consequently, these responses to bezafibrate led us to classify the various genotypes as "mild" (groups 2 and 3) or "severe" (group 1). We then analyzed these data to determine whether contributions of individual mutant alleles to the response to bezafibrate could be identified.

Cross-Analysis of Genotypes: Can the Response to Bezafibrate Predict the Severity of VLCAD Gene Point Mutations?

Group 1 includes four homozygous genotypes with point mutations N122D (genotype 6), G222R (genotype 12), R453Q (genotype 31), and R469W (genotype 33) and five heterozygous genotypes with a null mutation on one allele (nonsense, deletion, or frameshift leading to a premature stop codon) associated with one of the following point mutations on the other allele: G185S (genotype 10), T260M (genotype 16), R286G (genotype 1), G441D (genotype 15), or R613W (genotype 14). These genotypes identify nine distinct amino acid substitutions in the VLCAD protein sequence that lead to extremely low residual capacities and therefore seriously affect the activity and/or the stability of the mutant protein. In keeping with this, studies in cells of genotypes 6, 12, and 1 revealed undetectable VLCAD enzyme activities after treatment with bezafibrate. Genotype 11 (G185S/G294E) is the only compound heterozygous genotype in group 1; it included the severe G185S substitution already found in association with a null mutation (genotype 10, group 1). This indicates that expression of the allele carrying the G294E substitution cannot compensate for the effects of the G185S mutation and, consequently, that G294E is also a severe mutation.

Genotypes ranked in group 3 demonstrated a good response to bezafibrate, resulting in the restoration of FAO rates to control values even though most of these cell lines exhibited a significant FAO deficiency before treatment. Three of these cell lines carried the homozygous point mutations V174M (genotype 9), K264E (genotype 17), and V283A (genotype 20), indicating that these muta-

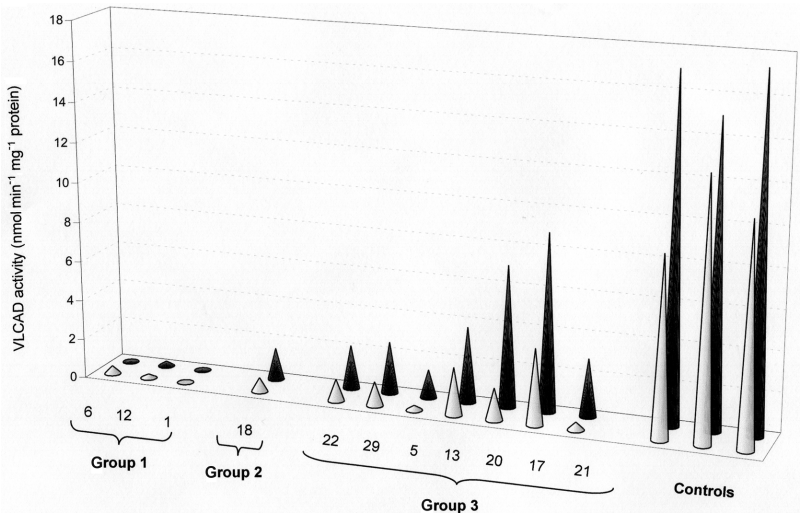

Figure 4. VLCAD activity measured in fibroblasts of VLCAD-deficient patients before (gray triangles) and after (black triangles) treatment with bezafibrate (400 μM for 48 h). Each determination was performed in duplicate.

tions were relatively benign amino acid substitutions. The V283A mutation was also found to be compound heterozygous in two group 3 genotypes associated either with G441D (genotype 21), a severe group 1 mutation, or with E462K (genotype 22), a well-known severe mutation that targets the catalytic residue of VLCAD.[32] Interestingly, after treatment with bezafibrate, enzyme activity determinations in fibroblasts from genotypes 20, 21, and 22 revealed similar activity levels in genotypes 21 and 22, which harbored one V283A allele, and about twice this level in genotype 20, which was homozygous for this mutation. This clearly suggests that the expression of a single allele carrying the V283A mutation is sufficient to restore normal FAO capacities in response to bezafibrate and can compensate for a highly deleterious mutation on the other allele. The K264E mutation present at a homozygous state in genotype 17 was also found to be compound heterozygous in genotypes 18 and 19. Interestingly, cells carrying the mutation K264E in association with the null mutation A333fsX358 (genotype 18, group 2) exhibited only a partial response to drug treatment, suggesting that the expression of a single allele carrying the K264E substitution leads to a variant enzyme that is functional but probably less active and/or stable than that in the case of V283A. On the other hand, the genotype K264E/M437V (genotype 19) exhibited FAO values that ranked among the highest observed in group 3, suggesting that the M437V allele could also contribute to the response to the drug and might therefore represent a mild mutation. Finally, as observed for V283A, a compensatory effect of a mild mutation over a severe one was suggested in the case of E504D. Indeed, genotype 13, associating E504D with the severe mutation G222R, exhibited high basal and drug-stimulated FAO rates, and enzyme levels measured in this cell line were consistent with these observations, clearly indicating that E504D alone can compensate for the presence of the severe G222R allele.

The genotypes 4, 26, and 30, carrying the missense mutations P89S, R366H, and A416T, respectively, were seen in combination with different null mutations. These three cell lines clearly ranked in group 2, suggesting that the P89S, R366H, or A416T substitutions, although relatively mild, are not compatible with restoration of full FAO capacities after treatment with bezafibrate. Two cell lines (genotypes 7 and 25 in groups 2 and 3, respectively) harbored the same substitution, K382Q, in combination with two distinct null mutations. The drug-stimulated FAO levels appeared slightly higher in genotype 25 compared with genotype 7, but there was overlap between these two cell lines, and these results clearly indicated that K382Q can be considered a relatively mild amino acid substitution. Interestingly, genotype K382Q/G447R (genotype 27, group 3) exhibited FAO rates quite similar to those found in genotype 25, in which K382Q is associated with a null allele. This suggests that the G447R substitution does not contribute to a significant extent to the response to bezafibrate and could therefore represent a severe mutation.

Altogether, cross-analysis of these genotypes led to the identification of a number of VLCAD mutant alleles that could each account for the observed FAO increase in response to bezafibrate. At the amino acid level, substitutions identified as mild were P89S, V174M, K264E, V283A, R366H, K382Q, A416T, M437V, and E504D. We also identified the severe mutations N122D, G185S, G222R, T260M, R286G, G294E, G441D, G447R, R453Q, R469W, and R613W. Finally, for some compound heterozygotes ranked in groups 2 or 3, analysis of our data alone could not predict the possible contributions of the individual alleles. This was the case for genotypes 5 (P91Q/G193R), 8 (S148G/R511W), 23 (K299M/L502Q), 24

(A304T/G439D), 28 (D405H/R450H), 29 (A416T/ R450H), and 32 (R456H/R615Q).

The mutation classification based on the response to bezafibrate was compared with functional data from the literature, and some representative mutations were located spatially on a three-dimensional model of the VLCAD protein (fig. 5). To our knowledge, no functional data are available for the severe point mutations identified in our experiments, with the exception of T260M, R469W, and R613W, as well as the double point mutation P65L+K247Q, for which previous reports demonstrated a complete absence or a very low residual VLCAD enzyme activity, consistent with our observations.[24,27,33] When mapped on the three-dimensional VLCAD model (fig. 5), a number of severe point mutations identified by our FAO test pointed to amino acid residues essential for the architecture of the active site (G185S), for flavin adenine dinucleotide (FAD) binding (G222R, G441D, and R453Q), or for protein stability (N122D, R286G, and G447R). In the case of G185S, for example, the mutation involves a residue immediately next to the conserved Ile184 lining the substrate-binding cavity that accommodates the fatty acyl moiety in the MCAD structure.[30,34] The mutation G222R involves the first residue of a conserved Gly-Ser-Asp segment directly involved in the binding of FAD in both MCAD and SCAD.[30,34,35] Similarly, the mutation G441D affects an amino acid that is part of a highly conserved Gly-Gly-x-Gly motif close to the FAD binding pocket.[17] In the case of R453Q, a mutation of the equivalent residue in SCAD (R359C) was shown to be disease causing in humans, and cDNA expression studies of this SCAD mutation suggested major protein-folding alterations.[36] Indeed, R453Q involves a highly conserved residue located near the two-fold axis of the VLCAD dimer and on one of the α-helices that form the FAD binding pocket domain of the enzyme.

Concerning the mild mutations characterized in our study, cDNA functional analysis of P89S, K264E, K382Q, A416T, and M437V VLCAD mutants in human fibroblasts[25] or Chinese hamster ovary cells[24] showed that all these variant proteins, though unstable, exhibited variable levels of residual enzyme activity. Similarly, the mutant V283A protein produced by cDNA expression in COS-7 cells exhibited significant residual activity, whose level appeared proportional to the amount of mutated protein.[5,37] The mild mutations identified through the FAO studies did not involve residues essential for enzyme catalysis, as assessed from the three-dimensional VLCAD model.

Discussion

Patients with VLCAD deficiency can present quite variable symptoms associated with a wide variety of gene mutations, but the molecular basis of disease variability is poorly understood. In particular, a large proportion of patients harbor gene missense mutations whose effects on residual metabolic capacities are often unknown.[5] The present study shows that studying the pharmacological profile of VLCAD-deficient cells can provide valuable information in comparing the relative severity of various genotypes or of missense mutations and, at the same time, can provide a genetic basis for a therapeutic approach to this disorder. Assays of palmitate oxidation revealed that about two-thirds (63%) of the patient cell lines exhibited a marked increase in their FAO capacities in response to bezafibrate, whereas, in the remaining cell lines, drug treatment was without effect. These differences could not be explained by variable inductions of VLCAD gene expression by bezafibrate, since similar increases in VLCAD mRNA levels were observed in all the treated cells, with the exception of those carrying null mutations leading to mRNA degradation (genotype 2). Interestingly, the lack of response to bezafibrate in genotype 2, and in two other cell lines carrying null mutations on both alleles (data not shown), indicates that a possible induction by bezafibrate of other β-oxidation enzymes, like the long-chain acyl-CoA dehydrogenase or the recently described acyl-CoA dehydrogenase 9,[38] does not contribute to the FAO capacities in the treated VLCAD-deficient fibroblasts. In fact, the pattern of FAO responses to bezafibrate appeared to be mainly determined by the variable degree of increase in VLCAD residual enzyme activity that resulted from the treatment. It could therefore be envisaged that the FAO values measured in drug-treated cells represent the end result of the variable consequences of the mutations on cell residual enzyme activity.

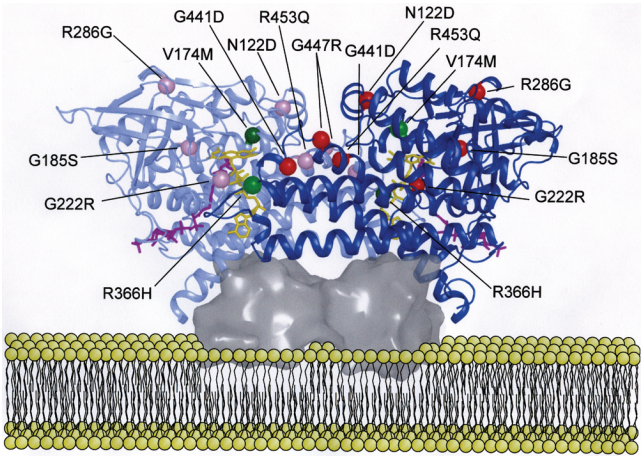

Figure 5. The dimeric structure of VLCAD shown with some human mutation sites. Each subunit is represented in light or dark blue. The FAD cofactor and the substrate (C8-CoA) are shown with sticks in yellow and pink, respectively. Mutation sites are shown in both subunits. Mutations on the light-blue monomer are marked with pink (severe) or dark-green (mild) balls, and those on the dark-blue monomer are shown with red (severe) or light-green (mild) balls. The C-terminal domain, which is unique only in VLCAD and is not present in other acyl-CoA dehydrogenases, is depicted as a gray blob anchored to the mitochondrial inner membrane.

This study consistently suggests that the various VLCAD gene mutations are the main determinants to explain the different responses to bezafibrate. In support of this, our results showed a complete restoration of FAO capacities in bezafibrate-treated cells carrying one or two copies of the V283A mutant allele, in keeping with previous studies showing that this mutant VLCAD enzyme retained a significant residual enzyme activity.[17,37] Conversely, negligible FAO rates were found in association with severe mutations like R613W, which was previously shown to fully abolish residual enzyme activity.[24] Since VLCAD is a dimeric enzyme, a functional complementation between two monomers carrying distinct mutations could be envisaged. For example, a missense mutation targeting the catalytic site might conceivably complement another missense mutation that does not affect catalysis but induces protein instability. However, careful analysis of the enzymatic and FAO data obtained from our panel of genotypes does not yield examples in support of this hypothesis. Cross-analysis of genotypes with common missense mutations always gave consistent pictures of the contribution of individual mutant alleles to the response to bezafibrate. It should also be mentioned that similar FAO responses were found when the effects of bezafibrate were compared in fibroblasts from several siblings or in cells from unrelated patients with identical genotypes (data not shown). Altogether, the response to bezafibrate is directly determined by the genotype.

In this regard, the results highlighted a first group of genotypes with missense mutations affecting amino acid positions often essential for enzyme catalysis, leading to a collapse of FAO capacities with no response to bezafibrate. This analysis showed a striking correlation with the presentation of the disease, since all the cells from patients with a severe neonatal or infantile form of the disease were finally classified in this group 1, which, in addition, included one single cell line from a patient with a myopathic phenotype but with an unusually severe presentation. Conversely, the same approach led to identification of the group 2 and group 3 genotypes, which exhibited a significant response to bezafibrate, and all these cell lines originated from patients with the myopathic disease phenotype. Analysis of the mild mutations in the three-dimensional model illustrated substitutions that generally did not disrupt bonding arrays essential for enzyme catalysis or quaternary structure but rather induced misfolded unstable proteins. Partial correction of FAO observed in some cases (group 2) often corresponded to genotypes with a missense mutation associated with a null mutation on the other allele. We hypothesize that some of these group 2 missense mutations might have generally more-deleterious effects than those found in the group 3 genotypes. This is illustrated in the case of R366H (genotype 26), which is predicted to weaken FAD binding and is associated with a relatively low FAO response after treatment with bezafibrate.

It can be concluded that measurements of FAO after pharmacological stimulation by bezafibrate provide a simple functional assay to optimally compare the residual metabolic capacities in a large panel of VLCAD-deficient cells. This assay allowed the identification of drug-responsive genotypes and, in some cases, drug-responsive individual mutations in this panel of genotypes. Thus, the correlation of pharmacological and genetic information allows the characterization of the effects of bezafibrate as a function of patient genotype. The FAO test in bezafibrate-treated cells also provides an index of the severity of metabolic block, which appears well correlated to the disease phenotype. This clearly suggests that the phenotypic variability is related to marked differences in the residual capacities of the β-oxidation pathway and provides new insights into the genotype-phenotype correlations in patients harboring missense mutations. We propose that this experimental approach could find valuable applications to improve the diagnosis and to allow better predictions of disease severity in relation to the genotype, an issue particularly important for the management of asymptomatic newborns.

Our results highlight the potential of bezafibrate to improve or correct the long-chain FAO defect in the patient cells. Indeed, treatment with bezafibrate significantly improved the FAO capacities in all the cells coming from patients with a typical myopathic form of the disease and restored normal FAO rates in the majority of these cell lines. So far, therapeutic approaches are extremely limited in this disease, as in many other FAO disorders.[8] The results obtained in the present study provide grounds for future clinical studies of a possible correction of VLCAD deficiency by bezafibrate, a drug that has been prescribed for >25 years with a good safety profile,[15] or for the use of some related compounds like new-generation PPAR agonists. The responsiveness to these drugs in relation to the genotype, which can be simply addressed in patient fibroblasts, provides a rationale to select responder genotypes, which could be used for the stratification of patients in future clinical trials. Finally, this study points out that, despite the great diversity of possible consequences of missense mutations for enzyme synthesis, activity, or steady-state level,[7] the pharmacological stimulation of mutant VLCAD gene expression turned out to improve or correct the β-oxidation capacities in a relatively large panel of genotypes. Altogether, integration of molecular information in a therapeutic strategy could find fruitful applications to develop new treatments adapted not only to specific mutations but also to a broad spectrum of genotypes.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for protein sequences of human [accession number P49748] and rat [accession number P45953] VLCAD; human [accession number P11310], pig [accession number P41367] and rat [accession number P08503] MCAD; and human [accession number P16219] and rat [accession number P15651] SCAD)

Multi-Align Tool, http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for VLCAD deficiency)

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