Bezafibrate increases very-long-chain acyl-CoA dehydrogenase protein and mRNA expression in deficient fibroblasts and is a potential therapy for fatty acid oxidation disorders

F. Djouadi1, F. Aubey1, D. Schlemmer1, J.P.N. Ruiter2, R.J.A. Wanders3, A.W. Strauss3 and Jean Bastin1,*

1INSERM U393, Hôpital Necker-Enfants Malades, Paris 75015, France, 2Laboratory for Genetic Metabolic Diseases, Academic Medical Centre, 1105AZ Amsterdam, The Netherlands and 3Vanderbilt Children’s Hospital, Nashville, TN 37232, USA

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Inherited defect in very-long-chain acyl-CoA dehydrogenase (VLCAD), a mitochondrial enzyme catalyzing the initial step of long-chain fatty acid β-oxidation (FAO), is one of the most frequent FAO enzyme defects. VLCAD deficiency is associated with clinical manifestations varying in severity, tissue involvement and age of onset. The molecular basis of VLCAD deficiency has been elucidated but therapeutic approaches are quite limited. In this study, we tested the hypothesis that fibrates, acting as agonist of peroxisome proliferator-activated receptors (PPARs), might stimulate FAO in VLCAD-deficient cells. We demonstrate that addition of bezafibrate or fenofibric acid in the culture medium induced a dose-dependent (up to 3-fold) increase in palmitate oxidation capacities in cells from patients with the myopathic form of VLCAD deficiency, but not in cells from severely affected patients. Complete normalization of cell FAO capacities could be achieved after exposure to 500 μM bezafibrate for 48 h. Cell therapy of VLCAD deficiency was related to drug-induced increases in VLCAD mRNA (+44 to +150%; P < 0.001), protein (1.5–2-fold) and residual enzyme activity (up to 7.7-fold) in patient cells. Bezafibrate also diminished the production of toxic long-chain acylcarnitines by 90% in cells harboring moderate VLCAD deficiency. Finally, real-time PCR studies indicated that bezafibrate potentially stimulated gene expression of other enzymes in the β-oxidation pathway. These data highlight the potential of fibrates in the correction of inborn FAO defects, as most mutations associated with these defects are compatible with the synthesis of a mutant protein with variable levels of residual enzyme activity.

INTRODUCTION

Mitochondrial β-oxidation of long-chain fatty acids is the main energy-producing pathway in many organs and tissues. Therefore, inherited defects in enzymes of mitochondrial fatty acid oxidation (FAO) are associated with a spectrum of clinical manifestations varying in severity, tissue involvement and age of onset (1). Very-long chain acyl-CoA dehydrogenase (VLCAD) is one of the four acyl-CoA dehydrogenases with various chain length specificities that catalyze the initial step of mitochondrial FAO. In humans, VLCAD is the main isoform involved in the oxidation of long-chain fatty acids. In fact, patients presenting with palmitoyl-CoA dehydrogenase deficiency by enzyme assay were discovered to have disease-causing mutations in the VLCAD gene and not in the long-chain acyl-CoA dehydrogenase (LCAD) gene (1). Like many FAO enzymes, VLCAD is ubiquitously expressed at various levels in most tissues. Nonetheless, VLCAD deficiency can manifest in quite different phenotypes (2), consistent with the clinical, biochemical and genetic variation.

*To whom correspondence should be addressed at: INSERM U393, Hôpital Necker-Enfants Malades, 149, rue de Sèvres, 75015 Paris, France. Tel: +33 144495161; Fax: +33 147348514; Email: bastin@necker.fr

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diversity of inborn FAO disorders. Furthermore, VLCAD deficiency is one among the more common FAO defects, with more than 100 reported patients (3). Three distinct presentations of the disease have been described. In its most severe form, VLCAD deficiency presents with severe neonatal cardiomyopathy and liver failure, generally leading to death. In the infantile onset form, delayed disease onset is encountered, characterized by hypoketotic hypoglycemia. Finally, a last group of VLCAD-deficient patients are asymptomatic early in life, but develop a purely myopathic form of the disease in adolescence or adulthood, with myalgia and exercise intolerance, usually with episodes of rhabdomyolysis triggered by metabolic stress conditions such as exercise, starvation, exposure to cold or fever; this presentation represents the largest group among all the VLCAD-deficient patients (3).

The molecular basis of VLCAD deficiency has been studied in a relatively large panel of patients. At present, more than 80 different gene mutations have been characterized that span the entire gene. No prevalent mutation has been identified among symptomatic patients (3). Besides its usefulness in family studies and prenatal diagnosis, mutation analysis defined clear genotype–phenotype correlations (2–4) because individuals with the severe phenotype generally carry gene deletions, insertions or mutations leading to complete lack of mRNA and/or protein expression, so-called null mutations. In contrast, the majority of VLCAD gene mutations found in the milder phenotypes are missense or small in-frame deletions or insertions compatible with mRNA production, translation and expression of some residual enzyme activity.

The correlation between disease phenotype and the extent of metabolic block has mainly been addressed by studies of acylcarnitines profile in blood or cultured cells of VLCAD-deficient patients (5,6). In fact, the development of electrospray ionization tandem mass spectrometry (ESI-MS-MS) has provided a powerful tool to characterize the patterns of acylcarnitine species that typically accumulate in various forms of the disease, secondary to impaired FAO. Altogether, these studies point out that the most severe phenotypes are associated with a more marked accumulation of long-chain acylcarnitines when compared with milder forms (7), and support the notion that residual long-chain FAO flux is extremely low in cells harboring a severe VLCAD defect, whereas milder phenotypes exhibit less profound defects in FAO capacities. In several American and European centers, large-scale applications of ESI-MS-MS have allowed systematic population-based neonatal screening for VLCAD deficiency (8). Combined with genetic analysis, this approach has greatly improved detection of this potentially severe FAO disorder. In particular, it is now possible to identify asymptomatic patients carrying disease-causing VLCAD gene mutations (9), long before the onset of clinical manifestations.

These rapid advances in the characterization of the disease over the last years are in marked contrast with the limited progress in therapy for VLCAD deficiency and FAO disorders. Management of patients is still essentially supportive, based on preventing hypoglycemia and conditions associated with mobilization of fatty acids as energy substrates (10) by avoidance of fasting. In the only therapeutic study (11), three patients with the severe form of VLCAD deficiency were apparently successfully treated by dietary supplementation with the seven-carbon medium-chain fatty acid, triheptanoin. However, no treatment has been described for the more frequent myopathic form of the disease. Overall, clinical heterogeneity of this and other FAO defects suggests that many different pathogenic mechanisms might be activated in response to the deficiency of a single enzyme step, making the design of an appropriate therapeutic strategy complex.

The clear genotype–phenotype correlation suggests that the pattern of tissue involvement and associated clinical manifestations depend, at least partially, on the levels of residual long-chain FAO flux and VLCAD protein expression in various tissues. This latter could be determined not only by intrinsic factors such as the nature of the mutation and stability of the mutant protein (3), but also by regulatory factors that control cell and tissue-specific VLCAD gene expression. Recent data highlight the importance of transcriptional regulation in the control of mitochondrial FAO (12). This regulatory network involves the nuclear hormone receptor family of peroxisomal proliferator-activated receptors (PPARs), which include the α, β/δ and γ isoforms. PPARα is preferentially expressed in tissues with high fatty acid utilization like heart and muscle, the PPARδ–β isoform is ubiquitously expressed and PPARγ is found at high levels in adipocytes and other cell types involved in lipid synthesis and storage (12). These nuclear hormone receptors act by coordinately controlling gene transcription rates of various enzymes in specific metabolic pathways. Thus, PPARα controls basal levels of FAO enzyme gene expression, can drive FAO gene adaptations to normal or pathological challenges and, therefore, plays a crucial role in energy homeostasis (13).

PPARs have attracted attention in terms of therapeutic potential because many ligands or activators of these receptors have been identified, including common drugs like fibrates (13). In fact, fibrates and other newly developed PPAR agonists are potent pharmaceutical tools to stimulate FAO in a wide variety of cells, tissues or animal models, as abundantly documented in the literature (12,13). FAO control by PPARs has stimulated active research in many different fields of medicine, including treatment of diabetes and obesity (14,15), but, surprisingly, has received no recognition for possible therapy of inborn FAO errors.

In the present study, we, therefore, investigated the effects of bezafibrate, a common hypolipidemic drug acting as a PPAR agonist, on FO capacities of VLCAD-deficient patients and provide data in support of a possible complete correction of this inborn FAO defect in response to treatment by this drug.

RESULTS

Bezafibrate increases palmitate oxidation in fibroblast cultures from VLCAD-deficient patients

Fibroblasts cultured from VLCAD-deficient patients were used to determine the effects of fenofibrate acid and bezafibrate, two widely used hypolipidemic drugs, on 3H-palmitate oxidation. Three patients (patients 1–3) carried missense mutations (see Materials and Methods) compatible with the milder, later-onset, myopathic form of the disease with episodic muscle weakness, myalgia and myoglobinuria (3). Patient
4 suffered from the severe form of the disease and had died suddenly in infancy. These phenotypes are in agreement with the residual palmitate oxidation measured in the patients' fibroblasts (Fig. 1). That is, patients 1–3 exhibited 33–48% of control palmitate oxidation values, but patient 4 had <10%. Similar results were obtained when the assay was run using 3H-oleate (data not shown).

Fibroblasts were treated with 100 μM fenofibric acid or 500 μM bezafibrate for 48 h. The optimal concentrations of fenofibric acid and bezafibrate were established by a dose–response experiment ranging from 50 to 500 μM (Fig. 1B and C). Exposure to fenofibric acid induced no significant changes in FAO in control and patient 4 fibroblasts. However, palmitate oxidation of fibroblasts from patients 1–3 treated with fenofibric acid was 1.8-, 2- and 1.5-fold greater than that in vehicle-treated cells. In contrast to fenofibric acid, bezafibrate resulted in an increase of FAO in control fibroblasts (+51%, P < 0.001). Response of patient 4 fibroblasts to bezafibrate was similar to fenofibric acid, with no significant change in FAO. However, in fibroblasts from patients 1–3, bezafibrate induced a 2.8–3.2-fold stimulation of palmitate oxidation rates, resulting in the restoration of normal palmitate oxidation.

**Bezafibrate corrects acylcarnitine accumulation in VLCAD-deficient fibroblasts**

As mentioned earlier, detection of abnormal concentrations of various acylcarnitines in body fluids (8) or cultured human cells (7) has become a common tool for biochemical diagnosis of inborn errors of FAO. The acylcarnitine profiles produced are characteristic and helpful in defining the specific enzyme defect in the FAO pathway.

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![Figure 1](image_url)

**Figure 1.** (A) Increases in palmitate oxidation in fibroblasts derived from normal or VLCAD-deficient individuals treated with fibrates. Cells were incubated for 48 h with 100 μM fenofibric acid, 500 μM bezafibrate or vehicle (DMSO). The assays were performed in triplicate, and the results are expressed as mean ± SD. ***P < 0.001 when compared with the DMSO-treated fibroblasts of a given subject. Dose–response studies of fenofibric acid (B) or bezafibrate (C) effects on tritiated palmitate oxidation rates, in control or patient 2 cells.
Fibroblasts from controls and VLCAD-deficient patients were incubated for 72 h with palmitate and l-carnitine (Fig. 2). Acylcarnitine intermediates detected by tandem mass spectrometry in VLCAD-deficient cells correspond to palmitoyl-(C16)-, myristoyl-(C14)-, dodecanoyl-(C12)-, decanoyl-(C10)- and octanoyl-(C8)- carnitines. The acylcarnitine profile from normal fibroblasts showed small peaks of C10 and C16 species (Fig. 2A).

In our VLCAD-deficient patients, and as reported by others (16,17), we demonstrated a more pronounced accumulation of C12-, C14- and C16-acylcarnitines in the severe phenotype (Fig. 2E) when compared with the milder form (Fig. 2C). Cells of patients 2 and 3 exhibited the same acylcarnitine profiles as patient 1 (data not shown).

The quantification of palmitoyl-(C16)-acylcarnitine (Fig. 2G) revealed that the concentration in cells from the severely VLCAD-deficient neonate was 1.5–3.5-fold higher than that in cells from the mild VLCAD-deficient individuals (patients 1–3), in agreement with data from others (16). In all cases, the production of C16-acylcarnitine in patient cells was 5–16-fold higher than that in control cells. Concerning the other acylcarnitine species, C14- and C12-acylcarnitines were found to be, on average, 6.5- and 8.8-fold higher or 2.3 and 3.8-fold higher in the mild or severe VLCAD-deficient fibroblasts, compared with control.

Because bezafibrate appeared to be more potent in stimulating FAO oxidation than fenofibric acid, we investigated the effect of bezafibrate on C16-acylcarnitine intermediates in control and VLCAD-deficient fibroblasts. The results indicate that a 3 day treatment of ‘mild’ VLCAD-deficient fibroblasts (patients 1–3) with bezafibrate normalized the C16-acylcarnitine production in mutant cells to control levels, but no beneficial effect was observed in severe VLCAD-deficient cells (patient 4). Bezafibrate also diminished the production of C14- and C12-acylcarnitines in both control and patient cells. Thus, the C14-acylcarnitines were decreased by 40% in control and moderately deficient fibroblasts, and by 53% in severely deficient cells. Concerning the C12-acylcarnitines, the decreases were more pronounced in all patient cells (~80%) than in control fibroblasts. Interestingly, although there was no abnormal accumulation of C8- or C10-acylcarnitine in the patient cells, bezafibrate decreased the amount of these acylcarnitines by ~75%.

Bezafibrate increases VLCAD enzyme activity

Fibroblasts from controls and VLCAD-deficient patients were treated with 500 μM bezafibrate for 48 h. In control cells, VLCAD enzyme activity was increased 2.2-fold, from 2.6 ± 0.2 to 5.8 ± 0.1, in response to bezafibrate (Fig. 3). The residual enzyme activity measured in mild-type VLCAD-deficient patient’s fibroblasts represented 15–20% of control values. Exposure of these cells to bezafibrate resulted in a highly significant rise in enzyme activity, ranging from +80% to +775% compared with vehicle-treated cells. In patients 2 and 3, bezafibrate actually restored enzyme activity to almost normal levels, whereas the value reached in patient 1 represented only ~35% of control values. Bezafibrate failed to increase VLCAD activity in cells from patient 4.

Bezafibrate increases VLCAD mRNA and other FAO enzyme mRNA

Quantitative RT-PCR analysis was performed to determine whether bezafibrate increased VLCAD gene expression. Treatment with 500 μM bezafibrate for 48 h (Table 1) resulted in stimulation (+44 to +150%; P < 0.001) of VLCAD gene expression in control and patient cells compared with untreated cells. Interestingly, bezafibrate also increased VLCAD mRNA in patient 4 cells.

Finally, we investigated the effect of bezafibrate on the mRNA levels of several other FAO pathway genes in control cells (Fig. 5). Mutations in each of these genes that cause FAO disorders have been described. In control cells, bezafibrate induced a significant increase (P < 0.001) in gene expression of each enzyme analyzed, ranging from +40% for ETF-A to +158% for CPT1-A.

DISCUSSION

The data presented in this paper provide the foundation for possible therapeutic intervention and pharmacological correction of VLCAD deficiency because three of four deficient cell lines harboring missense mutations of VLCAD gene were fully responsive to bezafibrate therapy. All patient’s fibroblasts initially exhibited reduced levels of 1H-palmitate oxidation, varying from 30 to 50% of control values in individuals with a relatively mild deficiency of VLCAD (patients 1–3) to <10% in patient 4 with a severe VLCAD deficiency. This functional impairment, together with the accumulation of long-chain acylcarnitines in the culture medium, is the hallmark of the metabolic block associated with VLCAD deficiency. Following 48 h of treatment with bezafibrate, both 1H-palmitate and acylcarnitine indexes were restored to normal in fibroblasts from patients 1–3, evidence for a strong stimulatory effect of the drug on FAO flux in these deficient cells. In contrast, fibroblasts from patient 4 failed to respond to bezafibrate treatment, based on the same criteria.

These differences in the response to bezafibrate did not correlate with the basal residual enzyme activities, because
Figure 2. Acylcarnitines profiles generated during incubation of human fibroblasts with l-carnitine and palmitate for 72 h. The signals represent molecular ions of acylcarnitine detected by tandem mass spectrometry from control cells (A and B), cells isolated from VLCAD-deficient patient with the mild muscle phenotype (C and D) or cells from the VLCAD-deficient patients with cardiomyopathy (E and F). Internal standards (IS): \( \text{[H}_3\text{]carnitine (m/z = 292), [H}_3\text{]myristoylcarnitine (m/z = 382), [H}_3\text{]palmitoylcarnitine (m/z = 404). (G) Quantification of C16 acylcarnitine. Determinations were performed in triplicate. Results are expressed as mean ± SD. **P < 0.001 when compared with the control. m/z, mass/charge.
we initially found equivalently low levels of VLCAD enzyme activity in the four untreated patient cell lines. Previous studies already suggested that residual VLCAD enzyme levels do not discriminate among the various disease phenotypes and that the low VLCAD enzyme activity is clearly related to a decreased amount of immunoreactive protein (2,5). In patients 2 and 3, bezafibrate raised VLCAD enzyme activity to the values normally found in control fibroblasts. Although stimulation of residual enzyme activity was more modest in patient 1, it was sufficient to restore normal FAO flux.

Western blot studies showed that these changes in enzyme activity could be explained by increased levels of immunoreactive VLCAD protein in response to bezafibrate. Therefore, the main beneficial effects of bezafibrate is an augmentation of steady-state levels of the variant VLCAD proteins, leading to enhanced residual VLCAD enzyme activity in the deficient cells. The relations between VLCAD mutant protein levels and residual enzyme activity have been previously analyzed by over-expression experiments (2). Interestingly, these studies focused on the effects of the V243A mutation, carried by patients 2 and 3 in our study. By transfection of the V243A mutant cDNA into COS-7 cells, it was shown that, in transfected cells, the levels of enzyme activity were proportional to the expression level of mutant VLCAD protein (2), and conclusions drawn from our study appear quite consistent with this notion.

Overall, our data clearly indicate that pharmacological correction of FAO capacities was related to parallel increases in VLCAD mRNA, immunoreactive protein and enzyme activity, triggered by drug treatment. It can be noted that in cells with severe enzyme deficiency (patient 4), bezafibrate increased VLCAD mRNA levels to the same extent as in cells of patients 1–3. Normal transcription of VLCAD gene is not the rule in patients with the severe presentation of the disease, who usually carry large deletions, premature stop codons or other null mutations leading to mRNA degradation (4). In our case, the molecular defects underlying VLCAD deficiency in different patients did not alter the transcriptional response to bezafibrate, but likely had variable consequences on the production of VLCAD protein under stimulation by this drug. It can be proposed that point mutations carried by patients 1–3 lead to accelerated degradation of the enzyme under basal conditions, accounting for the low residual VLCAD enzyme activity. However, increasing the rate of enzyme synthesis by pharmacological treatment probably compensates for this degradation in patient 1–3 cells, leading to increased residual VLCAD activity. In patient 4, in contrast, the amino acid substitution in VLCAD might dramatically affect folding, assembly and/or cellular handling of the enzyme, so that degradation cannot be compensated by increased enzyme synthesis.

Correction of VLCAD deficiency in patient fibroblasts very likely results from an activation of PPARα signaling pathway by bezafibrate. Indeed, although some reports initially suggested short-term inhibitory effects of bezafibrate on FAO (18), it is now firmly established that bezafibrate, acting as a PPAR agonist, has marked stimulatory effects on mitochondrial β-oxidation in many cell types of different species (13). Studies of PPARα gene knockout mice identified VLCAD as a target gene of this nuclear receptor. Thus, the basal level of VLCAD mRNA was reduced in several tissues or organs of PPARα −/− mice (19), and the induction of VLCAD by PPARα agonist or fibrates was also abolished in these knockout animal (19,20). Available data on the regulation of the human VLCAD gene failed to identify a nuclear hormone response element in the 5′ flanking regulatory regions of this gene (21). In addition to VLCAD, our results indicate that bezafibrate also stimulated the expression of several other FAO genes in human fibroblasts, including the carnitine palmitoyl-transferases (CPT1-A and CPT2), two acyl-CoA dehydrogenases (VLCAD and MCAD), the LCHAD which catalyzes the third step of long-chain FAO, the FAO electron transfer proteins (ETFa and ETFb) and the ETF dehydrogenase (ETF-QO) that is associated with the mitochondrial respiratory chain.

Inborn errors of the FAO pathway affecting each of these enzymes have been described. We speculate that bezafibrate could also be efficacious in correcting some of these other FAO disorders. In vitro studies in patient fibroblasts, such as those presented here, can easily be adapted to assess the effects of bezafibrate in other FAO enzyme defects. In fact, measurements of enzyme activity and FAO capacities, combined with studies of acylcarnitine profiles in treated cells should allow to determine whether bezafibrate can stimulate residual enzyme levels, and whether this has beneficial effects on β-oxidation flux in the deficient cells.

Our recent studies prove that bezafibrate can successfully correct the mitochondrial FAO defect in cells from patients presenting the mild forms of VLCAD or CPT2 deficiency (22), which both result in adolescent or adult-onset myopathy, and for which no therapeutic approach has been described. This represents a large group within the family of FAO disorders, with more than 200 affected patients described to date.
In conclusion, the potential for bezafibrate to correct inborn FAO disorders is highlighted in this study, and our results provide strong grounds for the design of clinical trials assessing the efficiency of this drug in CPT2 or VLCAD-deficient patients. Furthermore, our approach offers a valuable mean to select patients by in vitro studies before considering a clinical trial of bezafibrate. This pharmacological therapy opens new avenues for the treatment of FAO defects, based on a strategy that primarily targets the enzyme defect, rather than treatments directed at downstream consequences of the enzyme defect. Because the majority FAO defects are associated with missense mutations compatible with the synthesis of a mutant protein with variable levels of residual enzyme activity (4), this rationale is potentially applicable to many, but not all, FAO-deficient individuals. Furthermore, the ongoing development of new potent PPAR agonists for use in common metabolic diseases such as diabetes or obesity (15) may provide even better potential treatments for FAO disorders in the future.

Further screening of this class of drugs in different FAO disorders, with different genotypes and presentations, represents a new research track to address the legitimate demand for therapy of affected patients, augmenting progress made in understanding the origin and molecular basis of their diseases.

**MATERIALS AND METHODS**

**Patients**

Skin biopsies were performed from four VLCAD patients who carried the following mutations on VLCAD gene. Patient 1 was homozygous for the missense mutation, G520A, in exon 7 that predicts a V134M amino acid change. This patient was symptomatic, with episodic rhabdomyolysis following exercise and other symptoms typically associated with the adult-onset, myopathic form of the disease. Patients 2 and 3 were genotypically identical, with the same homozygous missense mutation, T848C, in exon 9 that predicts a V243A amino acid substitution. Both patients were detected by newborn screening and are asymptomatic on dietary management, although patient 3 does have mildly elevated serum CPK. Patient 4 died suddenly in infancy and has the homozygous, missense mutation, A364G, in exon 6 that predicts an N81D substitution. This baby had a severe clinical

![Figure 4](image-url). Effect of bezafibrate on VLCAD protein expression in fibroblasts from normal (A) and VLCAD-deficient individuals (B). Western blots of protein extracts from control and VLCAD-deficient cells were incubated with anti-VLCAD antibody and then re-probed with anti-β-actin antibody. (C) Histogram of VLCAD protein amount relative to β-actin. Values are depicted as mean ± SD of two independent experiments. **P < 0.01, ***P < 0.001 when compared with the vehicle-treated fibroblasts of a given subject.

<table>
<thead>
<tr>
<th>Table 1. Effects of bezafibrate on VLCAD gene expression</th>
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<tr>
<td><strong>Mean ± SE</strong></td>
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<td>Patient 3</td>
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<td>Patient 4</td>
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Total RNAs were isolated from human fibroblasts and quantified by quantitative RT-PCR as described in Materials and Methods. In each experiment, quantitative RT-PCR were run in duplicate. Control values were the mean of three different individuals.

*Gene expression levels in cells treated with 500 μM bezafibrate for 48 h relative to vehicle (DMSO) treated cells are presented from three different experiments.

***P < 0.001 when compared with DMSO-treated fibroblasts.
presentation of VLCAD deficiency. Informed consent was obtained from the parents of minors or from adult individuals.

Cell culture

Human skin fibroblasts from normal and VLCAD-deficient individuals were cultured in Ham’s F10 media (Invitrogen, Cergy-Pontoise, France) with glutamine, 12% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 0.1 mg/ml streptomycin and incubated in a humidified CO2 incubator (5% CO2, 95% air) at 37°C. For treatment, the media were removed, and the cells were subsequently incubated with fresh media containing bezafibrate (Sigma, Meylan, France) or fenofibric acid (kindly provided by FournierPharma, Dijon, France) or the equivalent amount of DMSO (vehicle) for 48 h.

Fatty acid oxidation

FAO was measured by quantifying the production of 3H2O from (9,10-3H) palmitate (Perkin Elmer, Courtaboeuf, France) as previously described in detail (23). The final concentration of palmitate was 125 μM, (60 Ci/mmol) bound to fatty acid-free albumin. We checked that the production of triitated water was linear with respect to time between 0 and 180 min in fibrate-treated and untreated cells as well. Palmitate oxidation rates by fibroblasts were expressed as nano-moles of 3H-fatty acid (3H FA) oxidized per hour per milligrams of cell protein (nmol 3H-FA h⁻¹ mg⁻¹ protein).

Enzyme activity

VLCAD activity was measured as described elsewhere (24) using palmitoyl-CoA as substrate. The production of 2,3-unsaturated-palmitoyl-CoA and β-hydroxypalmitoyl-CoA was quantified by HPLC and used to calculate acyl-CoA dehydrogenase activity.

Western blot analysis

Control and VLCAD fibroblasts were harvested in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) to prepare protein extracts. The lysates were centrifuged, and the supernatant was transferred to new vials. Protein concentration was determined using the Bradford method. About 20 μg of total protein per lane was resolved by 10% SDS–PAGE and transferred to Hybond-P PVDF membrane (Amersham Biosciences, Freiburg, Germany). The following antibodies were used: rabbit polyclonal anti-VLCAD and mouse monoclonal anti-actin (Chemicon International, Temecula, CA, USA). The primary antibodies were used at a dilution of 1:2000 and detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and the chemiluminescent reagent, ECL (Amersham Biosciences). Immunoreactive bands corresponding to the two protein antigens were scanned by densitometry with a computerized video densitometer. The results are characteristic of two independent experiments.

Real-time quantitative PCR

Total RNA was isolated by use of the Trizol reagent (Invitrogen), treated with DNase (Ambion, UK) and transcribed into cDNA. Real-time quantitative PCR (RT-PCR) was performed using the SYBR Green I kit and the LightCycler instrument from Roche Diagnostics (Mannheim, Germany). RT-PCR primers (Table 2) were designed using the sequences available in GenBank and spanned an intron/exon boundary. Gene expression levels were compared in fibroblasts treated with either vehicle or bezafibrate for 48 h. The amounts of the

Table 2. Primers used for real-time quantitative PCR

<table>
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<tr>
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<th>Reverse</th>
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Annealing temperature, 60°C.
various mRNAs were normalized to the amount of β-actin mRNA measured by quantitative RT-PCR in each sample. The results are in arbitrary units and expressed as fold changes in mRNA levels in cells treated with bezafibrate relative to vehicle-treated controls.

ESI-MS-MS-based acylcarnitine analysis

The method has been previously described in detail (25) with slight modifications. Briefly, at 100% confluency, fibroblasts were detached with trypsin, counted, plated (20 × 10⁵ cells per well in 24-well microplates) and settled to form a cell monolayer. The medium was then removed, and 1 ml of Ham’s F10 medium containing palmitate bound to BSA (4:1 molar ratio, 200 μM) and L-carnitine (400 μM) was added. After incubation at 37°C for 72 h, the reaction medium in each well was collected, and the cell monolayer was retained for protein quantification. The acylcarnitines in the reaction mixture were analyzed using an API3000 triple quadrupole mass spectrometer (Sciex, Applied Biosystem, USA) detecting the precursors of an m/z of 85 and quantitated by comparison with added, internal, deuterated standards.

Statistical analysis

Data are expressed as mean ± SD. Differences between vehicle and treated cells were analyzed by one-way analysis of variance (ANOVA) and the Fisher’s test; P < 0.05 was considered significant.

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Conflict of Interest statement. None declared.

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