Strategies for Correcting Very Long Chain Acyl-CoA Dehydrogenase Deficiency*

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Background: VLCAD deficiency is a mitochondrial fatty acid β-oxidation disorder.

Results: S-Nitrosylation of Cys-237 in VLCAD increased enzymatic activity and normalized β-oxidation capacity and acylcarnitine levels in VLCAD-deficient cells.

Conclusion: Correction of VLCAD deficiency alleviates disease-associated metabolic derangement and biomarker accumulation.

Significance: Data provide proof-of-concept for a potential therapeutic approach that may significantly impact the lives of children and adults with β-oxidation deficiencies.

Very long acyl-CoA dehydrogenase (VLCAD) deficiency is a genetic pediatric disorder presenting with a spectrum of phenotypes that remains for the most part untreatable. Here, we present a novel strategy for the correction of VLCAD deficiency by increasing mutant VLCAD enzymatic activity. Treatment of VLCAD-deficient fibroblasts, which express distinct mutant VLCAD protein and exhibit deficient fatty acid β-oxidation, with S-nitroso-N-acetylcysteine induced site-specific S-nitrosylation of VLCAD mutants at cysteine residue 237. Cysteine 237 S-nitrosylation was associated with an 8–17-fold increase in VLCAD-specific activity and concomitant correction of acylcarnitine profile and β-oxidation capacity, two hallmarks of the disorder. Overall, this study provides biochemical evidence for a potential therapeutic modality to correct β-oxidation deficiencies.

Mitochondrial β-oxidation of fatty acids (mFAO)³ is the main route for the metabolism of short, medium, and long chain fatty acids under normal, fasting, and metabolic stress conditions. Genetic defects of one or more proteins involved in mFAO result in a series of pediatric disorders collectively known as mFAO disorders, which are associated with a spectrum of clinical manifestations, including heart, liver, and muscular phenotypes (1–5). VLCAD deficiency is the second most common mitochondrial β-oxidation disorder (3–5). To date, more than 100 pathological mutations are known (1, 6–22), including null (typically associated with the most severe form of the disease) as well as missense mutations that occur throughout the VLCAD protein and are associated with the milder forms of the disease. Missense mutations result in reduced enzymatic activity and/or reduced stability of the protein leading to lower steady state levels of acyl-CoA dehydrogenase activity in mitochondria. Children with the most severe form of VLCAD deficiency present with cardiomyopathy and hepatic failure. This severe form is generally fatal in the 1st year of life (12–16, 22). A milder phenotype presents during early childhood with hypoketotic hypoglycemia and hepatomegaly without cardiomyopathy. The mildest phenotype is associated with later onset episodic myopathic form with intermittent rhabdomyolysis, muscle cramps, and/or pain and exercise intolerance (12–16, 22).

Upon diagnosis of VLCAD deficiency, emphasis is placed on the prevention of its manifestations. Currently, pharmacological treatments for the disease do not exist. Thus, preventive approaches include prevention of fasting and low fat formula with supplemental calories provided through medium chain triglycerides to bypass the metabolic block. Beyond this standard of care approach, an experimental oil called triheptanoin (a triglyceride with three heptanoic or seven carbon fatty acids esterified to a glycerol backbone) is under clinical trial to decrease muscle pain and to improve heart function (identifiers, NCT01379625 and NCT01886378). Moreover, bezafibrate, which is effective in most types of primary and secondary dyslipidemia, has been shown to restore FAO capacities in VLCAD-deficient patient fibroblasts by activation of the peroxisome proliferator activated receptor, leading to stimulation of residual enzyme activity (6).

VLCAD is one of the four acyl-CoA dehydrogenases catalyzing the first step of the mFAO. In mitochondria, two monomers of 71 kDa each are assembled to form the functional enzyme,
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Chemical Enrichment and Site-specific Identification of S-Nitrosothiols—Detailed experimental protocols for organic mercury resin-assisted capture and mass spectrometry-based site-specific identification of S-nitrosothiols have been published previously (25, 27, 28). Quantification of the fraction of S-nitrosylated VLCAD was based on Western blot analysis of bound and unbound fractions collected by mercury resin-assisted capture in cell lysates followed by densitometry-based quantification of the band corresponding to VLCAD (25, 28).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Palmitoyl coenzyme A lithium salt, ferricenium hexafluorophosphate, 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cGMP), and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were obtained from Sigma. Rabbit and goat (G-16 clone) polyclonal antibodies against VLCAD were obtained from Genetex (Irvine, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Rabbit and goat (K-19 clone) polyclonal antibodies against long chain acyl-CoA dehydrogenase (LCAD) were obtained from Genetex (Irvine, CA) and Santa Cruz Biotechnology, respectively. All chemicals and reagents used were of analytical grade.

Cell Culture and Treatments—Human VLCAD-deficient skin fibroblasts were provided by Dr. Bastin (INSERM U1124, Paris, France). Mutations G185S/G294E, N122D/N122D, and P91Q/G193R were identified in an individual having clinical symptoms consistent with the hepatic form of the disease characterized by hypoketotic episodes, hypoglycemia, and hepatomegaly (13, 26). Mutation P91Q/G193R was identified in an individual having clinical symptoms consistent with the hepatic form of the disorder accompanied by severe rhabdomyolysis (21). Human normal skin fibroblasts were obtained by Dr. Bastin and the American Type Culture Collection (ATCC). Cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 ng/ml streptomycin, at 37 °C in air with 5% CO₂. Cells were plated at a density of 10⁴ cells/cm² and cultured under normal conditions until they reached 90% confluence. The growth medium was replaced with serum-free RPMI 1640 medium, and freshly prepared S-nitroso-N-acetylcysteine (SNAC) or N-acetylcysteine (NAC) was added at final concentration of 100 μM. Thirty min later, SNAC or NAC was removed, and cells were extensively washed with PBS. Cell lysates were prepared into 250 mM Hepes, 1 mM diethylenetriamine pentaacetic acid, 0.1 mM N-ethylmaleimide, pH 7.7, 1% Triton X-100 and were protected from light exposure. Lysates were assayed for protein concentration, and equal amounts of protein per sample was used for organic mercury-assisted capture or other analyses.

Acylcarnitine Analysis in Normal and VLCAD-deficient Fibroblasts—Acylcarnitine levels were quantified by LC-MS/MS as described previously with some modifications (30). Cells at 90% confluence were exposed to NAC or SNAC for 30 min in serum-free medium. Then the cells were extensively washed with PBS, and fresh medium containing 0.2 mM palmitic acid and 0.4 mM 1-carnitine was added for 16 h. Cells were collected with trypsin, and acylcarnitines were extracted with brief sonication. Detection and quantification of acylcarnitine species were performed by LC-MS/MS using stable isotope-labeled internal standards (30).

Statistics—Comparisons between groups were performed using the unpaired t test.
**S-Nitrosylation Corrects VLCAD Deficiency**

![Dose-dependent effect of SNAC treatment on VLCAD enzymatic activity](image)

**FIGURE 1.** Dose-dependent effect of SNAC treatment on VLCAD enzymatic activity. Normal human skin fibroblasts were exposed to the indicated concentrations of NAC, NaNO₂, or SNAC for 30 min. VLCAD-specific activity was determined spectrophotometrically in cell lysates in the presence of 200 μM palmitoyl-CoA. 1 unit of activity is defined as the amount of enzyme that is required for reduction of 1 μmol of ferricenium per min. *, p < 0.01 (n = 3).

**RESULTS**

**SNAC Exposure Increases Mutant VLCAD-specific Activity**—To investigate whether site-specific S-nitrosylation would restore enzymatic activity of VLCAD in the presence of pathological mutations, VLCAD-deficient skin fibroblasts were exposed to SNAC or the parent molecule NAC. SNAC is known to enter cells and induce protein S-nitrosylation by transferring the nitric oxide equivalent to reduced cysteine residues of proteins (31).

A dose-dependent increase of VLCAD enzymatic activity, measured as palmitoyl-CoA dehydrogenation, was documented in normal fibroblasts treated with various concentrations of SNAC (Fig. 1). Maximal VLCAD activity was achieved in cells treated with 100 μM SNAC (Fig. 1). Exposure of fibroblasts to the precursors used for SNAC synthesis, NAC, and sodium nitrite (NaNO₂) did not alter VLCAD enzymatic activity (Fig. 1). To test for the specificity of the assay, cell lysates from NAC-treated normal fibroblasts and two SNAC-treated VLCAD-deficient cells were incubated with antibodies against VLCAD or LCAD for 20 min prior to the addition of palmitoyl-CoA. Incubation of cell lysates with antibodies against VLCAD reduced palmitoyl-CoA dehydrogenase activity by 93 ± 1% (Table 1). On the contrary, the inclusion of antibodies against LCAD did not affect the dehydrogenase activity. Moreover, LCAD protein was not detected by Western blot analysis in the same cell lysates, a finding that is in agreement with previous studies showing that LCAD is not detectable at the mRNA level nor at the protein level in human skin fibroblasts (32). Under these experimental conditions, LCAD protein was readily detected in mouse liver homogenates (data not shown). These results indicate that under the current experimental conditions the assay measures VLCAD-mediated dehydrogenation of palmitoyl-CoA. Exposure of fibroblasts to 100 μM SNAC up to 4 h did not induce cell death nor changes in morphology as assessed by the trypan blue exclusion test and light microscopy examination. Based on the SNAC dose-response curve, we selected to expose VLCAD-deficient cells to 100 μM SNAC. As a negative control, cells were exposed to 100 μM NAC.

Table 2 describes the VLCAD-deficient fibroblasts used in this study. These cells were derived from four patients diagnosed with the hepatic form of the disease. Compared with the wild type VLCAD, compound heterozygous mutations pG185S/G294E (patient 1) and pP91Q/G193R (patient 2) caused a 67% and 53% reduction of VLCAD protein levels, respectively. Homozygous mutation N122D/N122D (patient 3) caused an 81% reduction of VLCAD protein, and pP895/A536fsX550 mutation (patient 4) resulted in 83% reduction of VLCAD levels. As expected, the reduction in steady state levels of VLCAD protein was accompanied by significantly lower specific enzymatic activity as compared with normal cells (130 ± 20 milliunits/mg VLCAD, p < 0.001) upon NAC treatment (Fig. 2). VLCAD-specific activity did not correlate with VLCAD protein levels. For example, cells carrying the P91Q/G193R mutations expressed the highest levels of VLCAD protein among the panel of cells tested but exhibited the lowest specific activity (Table 2 and Fig. 2). Contrary to NAC treatment, which had no effect, a significant increase of mutant VLCAD-specific activity was measured in SNAC-treated cells (Fig. 2). The increase in enzymatic activity was not due to elevation of VLCAD protein levels upon SNAC treatment (Fig. 2, middle and bottom panel).

**SNAC Treatment Leads to Reversible S-Nitrosylation of Mutant VLCAD**—To gain insights into the mechanism of regulation of VLCAD enzymatic activity by SNAC, we initially explored the S-nitrosylation status of VLCAD in NAC- and SNAC-treated cells. Lysates were treated with methyl-methanethiosulfonate to block reduced cysteine residues and prevent them from reacting with organic mercury that was used for selective enrichment for S-nitrosylated cysteine residues in the cell lysates (27, 28). Then the methyl-methanethiosulfonate-blocked lysates reacted with phenyl mercury, which displaces nitric oxide and forms a covalent bond with cysteine thiol. Bound proteins were eluted with β-mercaptoethanol. Proteins were then probed with antibodies against VLCAD to identify S-nitrosylated VLCAD and quantify the modified fraction of the protein. Quantification was based on a standard curve that was generated using recombinant human VLCAD to titrate antibody binding.

Furthermore, organic mercury-assisted capture was employed for identification of site-specific S-nitrosylation by tandem mass spectrometry (27, 28). To this end, bound proteins were subjected to on-column digestion with trypsin. After extensive washes to remove unbound peptides, cysteine-containing peptides were eluted with performic acid. Mild performic acid cleaves the mercury–sulfur bond and oxidizes sulfur to sulfonic acid (SO₃H). This signature modification facilitates sequencing of peptides by MS/MS and simplifies post-MS data analysis (27).

VLCAD was detected in the unbound fractions of lysates from NAC- and SNAC-treated cells (Fig. 3A, upper panels). However, it was not detected in the bound fractions of lysates from NAC-treated cells (Fig. 3A, bottom left panel) indicative of...
TABLE 1
Contribution of VLCAD and LCAD on palmitoyl-CoA dehydrogenation
Cells were exposed to 100 μM NAC (normal cells) or 100 μM SNAC (VLCAD-deficient cells) for 30 min. Enzymatic activity was assessed spectrophotometrically in the presence of 200 μM palmitoyl-CoA. Anti-VLCAD or anti-LCAD antibodies were incubated with cell lysates for 20 min prior to the addition of palmitoyl-CoA. The table presents the average values with S.D. of three different experiments. The percentage of specific activity as compared with non-antibody-treated lysates is presented in parentheses.

| Mutations               | VLCAD μg/mg (n = 3) | Specific activity (milliunits/mg) |
|-------------------------|---------------------|----------------------------------|
| Normal + NAC           | 134 ± 7.0           | 11 ± 2.0 (8 ± 4%)*               |
| G185S/G294E + SNAC     | 124 ± 12            | 10 ± 2.0 (8 ± 2%)*               |
| P91Q/G193R + SNAC      | 106 ± 12            | 6.0 ± 2.0 (6 ± 2%)*              |

*p < 0.001 was compared with non-antibody-treated lysates.

TABLE 2
VLCAD-deficient skin fibroblasts used in the current study
VLCAD levels were quantified by Western blot analysis using human recombinant VLCAD for titration of antibody binding. Normal skin fibroblasts express 1.5 ± 0.12 μg of VLCAD per mg of lysate (n = 3).

| Mutations        | VLCAD μg/mg (n = 3) |
|------------------|---------------------|
| Patient 1        | 0.49 ± 0.08         |
| Patient 2        | 0.70 ± 0.01         |
| Patient 3        | 0.28 ± 0.07         |
| Patient 4        | 0.25 ± 0.04         |

FIGURE 2. SNAC treatment increases VLCAD-specific activity in VLCAD-deficient cells. Cells were exposed to 100 μM SNAC (gray columns) or NAC (white columns) for 30 min. Enzymatic activity was assessed spectrophotometrically in the presence of 200 μM palmitoyl-CoA. VLCAD protein levels are presented at the lower part of the figure. Note that SNAC treatment enhances enzymatic activity without altering protein levels. Basal VLCAD-specific activity in normal fibroblasts is 130 ± 20 milliunits/mg (n = 3). *p < 0.01 as compared with the corresponding NAC-treated cells.

FIGURE 3. SNAC treatment results in S-nitrosylation of VLCAD in VLCAD-deficient cells. A, cells were treated with 100 μM SNAC or NAC for 30 min. The cell lysates were then processed for mercury resin-assisted capture. Proteins in bound and unbound fractions were resolved by gel electrophoresis followed by Western blot-based identification of VLCAD. The immunoreactivity detected in the bound fractions of SNAC-treated cells was indicative of VLCAD S-nitrosylation in the original lysates. B, graph bar presenting the fraction of S-nitrosylated VLCAD detected in NAC-treated (shown as ND; not detected) and SNAC-treated (gray bars) cells.

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The absence of S-nitrosylated VLCAD in these cells. Robust immunoreactivity was evident in the bound fractions corresponding to VLCAD-deficient cells treated with SNAC (Fig. 3A, bottom right panel) indicating the presence of S-nitrosylated VLCAD in cell lysates. Using recombinant human VLCAD to titrate antibody binding, we quantified that on average 55 ± 10% of VLCAD molecules were S-nitrosylated in VLCAD-deficient cells treated with SNAC (Fig. 3B). Mass spectrometry was employed to investigate site-specific S-nitrosylation on VLCAD. No cysteine-containing peptide belonging to VLCAD was identified in lysates from NAC-treated VLCAD-deficient cells. An MS spectrum (Fig. 4A) assigned to the doubly charged peptide of human VLCAD TSAPSGKGLYKTLNGSK (underline indicates Cys-237) (Fig. 4B) was detected in all four VLCAD-deficient cells treated with SNAC, thus confirming the data obtained by Western blot analysis.

The canonical NO signaling pathway involves the activation of soluble guanylate cyclase (sGC) followed by the synthesis of cGMP and the activation of downstream cGMP-dependent kinases (33–35). To elucidate whether SNAC treatment activates the canonical NO-signaling pathway, which in turn impacts VLCAD activity, we treated VLCAD-deficient cells (mutation P91Q/G193R) with NAC, SNAC, and 8-Br-cGMP alone or with ODQ, an inhibitor of sGC (36), along with NAC or SNAC. Increasing the levels of cGMP by the addition of 8-Br-cGMP or inhibiting sGC activity by ODQ did not increase mutant VLCAD activity. However, a significant increase in VLCAD activity accompanied by robust S-nitrosylation was documented in cells treated with ODQ and SNAC or SNAC alone (Fig. 5). In addition, exposure of cell lysates (mutation P91Q/G193R) to UV light for 10 min prior to the addition of palmitoyl-CoA abolished the increased enzymatic activity by 93 ± 3%. Previous studies have shown that short term exposure to UV light effectively eliminates protein-bound NO forming a reduced thiol (37).

Furthermore, a time-dependent decline of VLCAD S-nitrosylation and enzymatic activity was documented in cell cultures (VLCAD P91Q/G193R cells) exposed initially to SNAC for 30 min followed by extensive wash and re-culture.
with fresh medium without SNAC (Fig. 6). Under these conditions, VLCAD protein levels did not change (data not shown) indicating that the time-dependent decline of $S$-nitrosylation and specific activity was not due to accelerated degradation of VLCAD. Collectively, these data suggest that the SNAC-mediated increase of VLCAD enzymatic activity occurs through a molecular mechanism that is independent from sGC activity and cGMP but involves reversible $S$-nitrosylation on cysteine 237.

**Correction of mFAO Capacity and Restoration of Acylcarnitine Levels in SNAC-treated Cells**—Low $\beta$-oxidation capacity and abnormal acylcarnitine profile represent markers for clinical diagnosis of VLCAD deficiency. Using $^3$H-labeled palmitate as substrate, we determined the $\beta$-oxidation rate by quantifying the release of $^3$H$_2$O in the growth medium. As expected, low oxidation rates (0.4–0.8 nmol/h/mg) were quantified in NAC-treated VLCAD-deficient cells, which was significantly lower than the rate measured in normal cells (2.6–$\pm$0.4 nmol/h/mg) (Fig. 7). Oxidation rates of 3.9–4.9 nmol/h/mg were quantified in SNAC-treated cells indicating a 6–11-fold increase of $\beta$-oxidation capacity (Fig. 7).

Moreover, using LC-MS/MS, acylcarnitine levels were quantified in normal as well as in cells carrying the mutations G185S/G294E and P91Q/G193R. As expected, elevated levels of saturated and unsaturated C12, C14, and C16 acylcarnitine species were quantified in VLCAD-deficient cells treated with NAC as compared with normal cells (Table 3). Interestingly, in SNAC-treated cells, significantly lower levels of all aforementioned species were quantified as compared with the corresponding NAC-treated cells (Table 3). When SNAC-treated cells were compared with normal fibroblasts slightly altered levels of C14:0 and C16:0 acylcarnitines were quantified in G185S/G294E cells. In addition, C14:0, C14:1, and C16:0 species were elevated in P91Q/G193R cells as compared with normal fibroblasts (Table 3). These data indicate that SNAC treatment corrected for the most part the metabolic abnormalities causing accumulation of acylcarnitine species. Overall, the data document site-specific $S$-nitrosylation of VLCAD resulting in a substantial increase of the specific activity in the presence of pathological mutations upon SNAC treatment. Corrected enzymatic activity contributes to the restoration of $\beta$-oxidation capacity and normalization of acylcarnitine levels. Although our study was performed in a cell model system, it provides initial evidence for the design of preclinical in vivo models that will explore the potential of site-specific $S$-nitrosylation toward the correction of VLCAD deficiency as well as other mitochondrial $\beta$-oxidation-related disorders.

**DISCUSSION**

VLCAD deficiency is an autosomal recessive genetic disorder that presents with a spectrum of clinical phenotypes. Typically, the milder phenotypes are associated with missense mutations on VLCAD affecting both the stability and enzymatic activity. Contrary to medium chain acyl-CoA dehydrogenase deficiency where 80% of the affected individuals carry the K304E mutation (38), VLCAD deficiency is highly heterogeneous with over 100 pathological mutations reported (6–22). Upon diagnosis of VLCAD deficiency, the standard of care approach aims to prevent disease manifestations because a therapeutic approach is not available.

Enzymatic activity is typically regulated by protein abundance, allosteric and post-translational modifications. Protein $S$-nitrosylation, the covalent modification of reduced cysteine residues by nitric oxide, represents a major mechanism by
which nitric oxide modulates signaling and regulates protein function (25, 39–48). Our previous work in mice has shown that S-nitrosylation of VLCAD on cysteine 238 increased catalytic efficiency by 29-fold and normalized β-oxidation capacity in vivo (25). Here, we explored whether this regulatory mechanism has the potential to correct enzymatic activity in the setting of VLCAD deficiency. To this end, we employed fibroblasts isolated from four individuals with documented VLCAD missense mutations. The mutations studied are not located on amino acids that participate in catalysis (462E) or nucleotide (366R) and substrate (223S, 463G) binding. It is unclear how these mutations not only lower the protein levels but also in the case of P91Q/G193R mutation lower the enzymatic activity.

Cells were exposed to SNAC an efficient trans-nitrosylating agent that enters cells through the L-amino acid transporter, a known transport mechanism for the parent compound NAC (31). Our data documented that SNAC treatment induced reversible S-nitrosylation of VLCAD on cysteine 237 and substantially increased its enzymatic activity without altering protein levels (Figs. 2, 3, and 6).

The crystal structure of human VLCAD has been resolved by x-ray crystallography (Protein Data Bank code 2UXW). Cysteine 237 is relatively exposed to the surface of the protein and is localized at a loop region, which typically represents flexible secondary structures. Using the crystallographic object-oriented kit (coot), which facilitates protein modeling (49), we confirmed that the secondary structure around cysteine 237 and its exposure to the surface of the protein are not affected by the mutations. Based on this evidence, we can infer that wild type and mutant VLCAD have similar propensity to S-nitrosylation. Consistent with previous observations in mice, tissue homogenates, and cells, molecules that are capable of trans-nitrosylation, such as SNAC and S-nitrosoglutathione, target the same cysteine (Cys-238/237) and no other cysteine residue(s) on VLCAD protein (25, 27). Cysteine 238 is the confirmed endogenous S-nitrosylation site in wild type mice (25, 27). However, the mechanism by which VLCAD trans-nitrosylation occurs, i.e. directly by SNAC or mediated by another protein, is unknown and requires further investigation.

Cysteine residue at position 237 (238 in mouse sequence) is known to be involved in mFAO. Under these conditions, mFAO is significantly increased to provide metabolic fuel to meet the energy demands (50–52).
S-Nitrosylation Corrects VLCAD Deficiency

Failure to adapt to this challenge results in metabolic disarrangements with significant health impact. Individuals with VLCAD deficiency develop disease manifestations under conditions where increased mFAO activity is required. VLCAD catalyzes the first step on the mFAO cycle. It accommodates the large volume of acyl-CoAs constraining the flow of dehydrogenated intermediates toward the next enzymes of the cycle (bottleneck phenomenon) and thus slowing down the entire pathway. Remarkably, normal mFAO rate was documented in SNAC-treated cells implying that upon increase of VLCAD enzymatic activity via S-nitrosylation the flow of acyl-CoA species through the first step of the pathway is restored and the overall performance of mFAO cycle is normalized.

Using our previously published mass spectrometry-based method for site-specific identification of S-nitrosylated proteins, we have identified nearly 200 modified proteins in VLCAD-deficient fibroblasts treated with SNAC. The enzymes carnitine palmitoyltransferases I and II, which catalyze the transfer and activation of long chain fatty acids in the mitochondria, were identified among the targets of SNAC-mediated S-nitrosylation. S-Nitrosylation does not inhibit the activity of these two critical proteins because after SNAC treatment the acylcarnitine species in VLCAD mutant cells were at normal or nearly normal levels as compared with controls and lower than the NAC-treated mutant VLCAD-expressing cells. Based on these findings, we suggest that S-nitrosylation of carnitine palmitoyltransferases I and II may also facilitate the activation of fatty acids to enter the mFAO pathway, and together with the correction of VLCAD enzymatic activity, it accounts for the correction in the levels of acylcarnitine species. Protein regulation through post-translational modifications represents a novel and promising area of investigation. Protein kinase, phosphatase, and histone deacetylase inhibitors have been designed, and their potential as anticancer drugs has been investigated in cell and animal models (53, 54). Here, studies in cells isolated from individuals with documented VLCAD deficiency show for first time that correction of enzymatic activity via site-specific S-nitrosylation improves biochemical and metabolic parame-

TABLE 3
SNAC treatment corrects acylcarnitine profile in VLCAD-deficient skin fibroblasts
VLCAD-deficient cells from patient 1 (mutation G185S/G294E) and patient 2 (mutation P91Q/G193R) were exposed to SNAC or NAC for 30 min in serum-free medium. Cells were extensively washed with PBS and re-cultured in growth medium containing 200 μM palmitoyl carnitine for 16 h. Acylcarnitine levels were quantified by LC-MS/MS using stable isotope internal standards. For comparison purposes, NAC-treated normal fibroblasts were also analyzed.

| Acylcarnitine | G185S/G294E (n = 3) | P91Q/G193R (n = 3) | Normal cells (n = 3) |
|---------------|---------------------|---------------------|---------------------|
|               | NAC | SNAC | NAC | SNAC | NAC |
| C₁₂           | 448 ± 90<sup>a</sup> | 201 ± 43<sup>a</sup> | 204 ± 28<sup>b</sup> |
| C₁₄           | 2190 ± 310<sup>a</sup> | 1050 ± 100<sup>a</sup> | 751 ± 52<sup>b</sup> |
| C₁₆           | 3730 ± 315<sup>a</sup> | 2600 ± 390<sup>a</sup> | 2710 ± 180<sup>b</sup> |
| C₁₂:1         | 41 ± 6.0<sup>a</sup> | 15 ± 3.0<sup>a</sup> | 10 ± 3.0<sup>a</sup> |
| C₁₄:1         | 108 ± 19<sup>b</sup> | 24 ± 6.0<sup>b</sup> | 22 ± 2.0<sup>b</sup> |
| C₁₆:1         | 77 ± 9.0<sup>a</sup> | 36 ± 4.0<sup>a</sup> | 55 ± 6.0<sup>b</sup> |
| C₁₂:2         | 22 ± 5.0<sup>a</sup> | 5.0 ± 2.0<sup>a</sup> | 4.0 ± 2.0<sup>a</sup> |
| C₁₄:2         | 44 ± 6.0<sup>a</sup> | 11 ± 3<sup>a</sup> | 9.0 ± 2.0<sup>a</sup> |
| C₁₆:2         | 17 ± 4.0<sup>a</sup> | 8.0 ± 2.0<sup>a</sup> | 9.0 ± 2.0<sup>a</sup> |

<sup>a</sup> Data indicate statistically higher values (p < 0.05) as compared with the corresponding SNAC-treated and normal cells.
<sup>b</sup> Data indicate a statistical difference (p < 0.05) versus normal fibroblasts.

FIGURE 7. S-Nitrosylation of VLCAD restores β-oxidation capacity in VLCAD-deficient cells. Cells were treated with 100 μM SNAC (gray bars) or the parent molecule NAC (white bars) for 30 min. Then the cells were cultured for 120 min in growth medium containing [3H]palmitate. Palmitate oxidation rate was determined by the quantification of [3H]H₂O released into the growth medium. For comparison, the corresponding rate in normal cells is presented. Each bar presents the average value ± S.D. of three independent experiments. *, p < 0.001 as compared with NAC-treated VLCAD-deficient cells.

Failure to adapt to this challenge results in metabolic disarrangements with significant health impact. Individuals with VLCAD deficiency develop disease manifestations under conditions where increased mFAO activity is required. VLCAD catalyzes the first step on the mFAO cycle. It accommodates the long chain acyl-CoAs entering the cycle for first time and also acyl-CoAs that have passed through the mFAO spiral already re-entering for further oxidation. In the setting of VLCAD deficiency, the residual enzymatic activity is not sufficient to accommodate the large volume of acyl-CoAs constraining the flow of dehydrogenated intermediates toward the next enzymes of the cycle (bottleneck phenomenon) and thus slowing down the entire pathway. Remarkably, normal mFAO rate was documented in SNAC-treated cells implying that upon increase of VLCAD enzymatic activity via S-nitrosylation the flow of acyl-CoA species through the first step of the pathway is restored and the overall performance of mFAO cycle is normalized.

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| C₁₂           | 448 ± 90<sup>a</sup> | 201 ± 43<sup>a</sup> | 204 ± 28<sup>b</sup> |
| C₁₄           | 2190 ± 310<sup>a</sup> | 1050 ± 100<sup>a</sup> | 751 ± 52<sup>b</sup> |
| C₁₆           | 3730 ± 315<sup>a</sup> | 2600 ± 390<sup>a</sup> | 2710 ± 180<sup>b</sup> |
| C₁₂:1         | 41 ± 6.0<sup>a</sup> | 15 ± 3.0<sup>a</sup> | 10 ± 3.0<sup>a</sup> |
| C₁₄:1         | 108 ± 19<sup>b</sup> | 24 ± 6.0<sup>b</sup> | 22 ± 2.0<sup>b</sup> |
| C₁₆:1         | 77 ± 9.0<sup>a</sup> | 36 ± 4.0<sup>a</sup> | 55 ± 6.0<sup>b</sup> |
| C₁₂:2         | 22 ± 5.0<sup>a</sup> | 5.0 ± 2.0<sup>a</sup> | 4.0 ± 2.0<sup>a</sup> |
| C₁₄:2         | 44 ± 6.0<sup>a</sup> | 11 ± 3<sup>a</sup> | 9.0 ± 2.0<sup>a</sup> |
| C₁₆:2         | 17 ± 4.0<sup>a</sup> | 8.0 ± 2.0<sup>a</sup> | 9.0 ± 2.0<sup>a</sup> |

<sup>a</sup> Data indicate statistically higher values (p < 0.05) as compared with the corresponding SNAC-treated and normal cells.
<sup>b</sup> Data indicate a statistical difference (p < 0.05) versus normal fibroblasts.
ters associated with impaired oxidation of long chain fatty acids within the mitochondria. Overall, this study represents a pre-
clinical screening of potential therapeutic modalities that will significantly impact the lives of children and adults with \( \beta \)-ox-
idation deficiencies.

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