

Exposure to resveratrol triggers pharmacological correction of fatty acid utilization in human fatty acid oxidation-deficient fibroblasts

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Carnitine palmitoyl transferase 2 (CPT2) and very-long-chain Acyl-CoA dehydrogenase (VLCAD) deficiencies are among the most common inborn mitochondrial fatty acid β -oxidation (FAO) disorders. Despite advances in their clinical and molecular characterizations, few therapeutic approaches exist for these diseases. Resveratrol (RSV) is a natural polyphenol extensively studied for its potential health benefits. Indeed, it is presently thought that RSV could delay the onset of some cancers, and have protective effects against common aging disorders such as type II diabetes, cardiovascular or neurodegenerative diseases. Here, we show that exposure to RSV induces a dose- and time-dependant increase in FAO flux in human fibroblasts, and can restore normal FAO capacities in a panel of patients' fibroblasts with the mild forms (harboring various genotypes) of CPT2 or VLCAD deficiency. The correction of FAO flux correlated with a marked increase in mutant CPT2 or VLCAD protein level, in cells treated by RSV. Inhibition of sirtuin 1 (SIRT1) by Sirtinol and the use of peroxisome proliferator-activated receptor gamma co-activator-1- α (PGC-1 α) small interfering RNAs demonstrate that the RSV-induced stimulation of FAO requires the presence of PGC-1 α and SIRT1. These results show, for the first time, that RSV markedly induces mitochondrial FAO capacities in human fibroblasts, and provides the initial proof-of-concept that RSV might be efficient for correction of inherited FAO disorders.

INTRODUCTION

Mitochondrial fatty acid β -oxidation (FAO) disorders form a large group of inherited metabolic diseases initially described more than 30 years ago (1,2). Among these, the carnitine palmitoyl transferase 2 (CPT2) and the very long chain acyl-CoA dehydrogenase (VLCAD) deficiencies are considered relatively common, and they share many characteristics (3,4). The CPT2 and VLCAD proteins are both located on the inner mitochondrial membrane. The CPT2 participates in the transfer of long-chain fatty acids into the mitochondria. The long-chain acyl-CoAs produced by CPT2 are the substrates of VLCAD, which catalyzes the initial step of the Lynen helix. A number of tissues, including the heart, the liver or skeletal muscle, are tightly dependent on FAO for energy production, and, accordingly, genetic enzyme defects in this metabolic pathway are generally associated with complex clinical manifestations. In line with this, CPT2 or VLCAD deficiency is associated with

several possible phenotypes, which vary in terms of severity, age of onset and the tissues affected (3,4). The most severe form of CPT2 deficiency is characterized by acute liver failure, hypoketotic hypoglycemia, peripheral myopathy and serious cardiac damage, generally fatal during the neonatal period. A severe neonatal phenotype has also been described in VLCAD deficiency, which associates hepatic failure and life-threatening cardiomyopathy. VLCAD deficiency can also manifest itself in infancy by isolated liver failure with episodes of hepatic coma. However, the most common phenotype of both disorders is an adolescent-onset myopathy. Typical manifestations of this metabolic myopathy are muscle stiffness, myalgia and recurrent attacks of rhabdomyolysis triggered by exercise, fever or exposure to cold. These muscular forms of CPT2 or VLCAD deficiency are generally considered as relatively mild, as long as life-threatening complications, such as acute renal failure induced by rhabdomyolysis, can be prevented (5).

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Both disorders have been extensively characterized at the molecular level, revealing a large number of disease-causing mutations in the CPT2 and VLCAD genes (3,4,6). Severely affected patients often harbor null-type mutations (large deletions, truncating, nonsense, etc.), which prevent the production of any protein, thus leading to the collapse of cellular FAO capacities and, therefore, accounting for the clinical severity of the associated phenotypes (3,7). However, and as observed in other FAO defects, the majority of gene variations found in CPT2- or VLCAD-deficient patients are missense mutations (8). In the mild presentation characterizing these disorders, patient cell studies have established that gene missense mutations often do not lead to a complete absence of the corresponding protein, but, rather, induce the production of a misfolded, unstable, mutant protein with some residual levels of protein and enzyme activity (7,9,10). Despite these advances in the characterization of CPT2 and VLCAD deficiencies, few therapeutic approaches exist for these disorders. To date, the management of patients remains essentially supportive (5,11,12). A high-carbohydrate fat-restricted diet, associated with the prevention of fasting or exercise, has been the mainstay of treatment for many years. These preventive measures, which alleviate muscular symptoms in some myopathic patients, do not, however, prevent the onset of muscular alterations, which are associated with CPT2 or VLCAD deficiency.

As mentioned above, the literature data consistently suggest that some degree of correlation exists between the severity of clinical manifestations and the extent of metabolic block as assessed in the patients' cells. Based on this, it can be surmised that treatments aimed at stimulating residual enzyme activity and FAO capacity might be of therapeutic value in CPT2 or VLCAD deficiency. In keeping with this hypothesis, we established that treatment by bezafibrate, a hypolipidemic drug known to activate peroxisome proliferator activated receptor (PPAR) transcription factors, can correct the FAO defect in cells from patients with the mild form of these diseases (9,13,14). Recently, a pilot trial of bezafibrate in myopathic CPT2-deficient patients suggested that this pharmacological approach could be successful *in vivo* (15,16).

These findings demonstrate that target-based therapy can be successful for the treatment of genetic FAO disorders, and prompted us to extend this paradigm to another key regulator of energy metabolism, namely the peroxisome proliferator-activated receptor gamma co-activator-1-alpha (PGC-1 α). PGC-1 α acts as a co-activator of numerous transcription factors [PPARs, NRF 1 and 2 (nuclear respiratory factors), hepatic nuclear factor 4], and is a master regulator of mitochondrial biogenesis and mitochondrial oxidative metabolism (17,18). Accordingly, PGC-1 α can be considered to be a highly relevant therapeutic target to stimulate residual metabolic capacities in FAO-deficient cells.

Recent studies have shown the importance of post-translational modifications in the control of PGC-1 α activity, and suggest that post-translational activation of PGC-1 α can be achieved by various classes of molecules, acting through different signaling pathways (18). In particular, PGC-1 α can be de-acetylated by sirtuin 1 (SIRT1), which results in PGC-1 α activation (19). SIRT1 is a member of the sirtuin family of nicotinamide adenine dinucleotide (NAD)-dependant histone deacetylases, and it is thought to be involved in the

adaptation of cell metabolism to nutritional changes, which it senses via changes in the levels of NAD⁺ (20). Another activation mechanism could involve phosphorylation of PGC-1 α , achieved by the adenosine monophosphate (AMP)-activated protein kinase (AMPK). Activation by phosphorylation might provide another way to link PGC-1 α activity to cell energy demand, since AMPK is considered to act as a fuel gauge activated by low-energy status (20).

Resveratrol (RSV) is a natural polyphenolic compound, first isolated from various plant roots. RSV is, probably, one of the most studied natural compounds, because of its pleiotropic biological properties. Indeed, it is presently thought that RSV could delay the onset of some cancers, and have protective effects against common aging disorders such as type II diabetes, cardiovascular or neurodegenerative diseases, by its possible action on the cell cycle, apoptosis or inflammation, and by its antioxidant properties (21). In addition, RSV has recently emerged as one of the most potent natural activators of SIRT1 (22–24), and various studies suggest that RSV might also act as an AMPK activator (25–27).

In this study, we investigated the potential of RSV to stimulate FAO capacities in a panel of patient's fibroblasts harboring CPT2 or VLCAD deficiencies, and we sought to delineate the molecular mechanisms underlying RSV metabolic effects in the patient cells.

RESULTS

RSV can restore normal FAO flux in a panel of FAO-deficient fibroblasts

In order to characterize the cellular response to RSV, palmitate oxidation rates were measured first in dose–response and time course experiments performed in control and VLCAD- and CPT2-deficient fibroblasts. The optimal concentration of RSV was established by dose–response experiments employing 10 to 125 μ M of the compound in cells treated for 48 h, as shown in Figure 1A. RSV induced a dose-dependant increase in palmitate oxidation rate in all the cell lines. In the patients' cells, the restoration of normal palmitate oxidation rates was observed at concentrations as low as 50 μ M RSV, and the maximal rates were reached at 75 μ M. At higher concentrations, RSV slowed down cell proliferation, as reflected by lower (–15 to –25%) protein levels. Based on these data, the effects of 75 μ M RSV were studied over a period of 12–72 h. These time course experiments revealed significant increases in FAO after 24 h of exposure, and maximal stimulation was reached at 48 h (Fig. 1B).

A panel of 13 VLCAD-deficient cell lines with different genotypes (Table 1) was then treated for 48 h with 75 μ M RSV (Fig. 1C). Two patients (Patients 1 and 2) suffered from the severe form of the disease and, accordingly, exhibited very low residual palmitate oxidation in fibroblasts (~5% of control values). In these severely deficient cell lines, RSV induced no significant change in FAO. In contrast, all the other patients (Patients 3–13) had been diagnosed with the milder, myopathic form of the disease and carried missense mutations that appeared compatible with the expression of low but significant residual FA oxidation (Fig. 1C). Indeed, under basal conditions, the fibroblasts from Patients 3–13

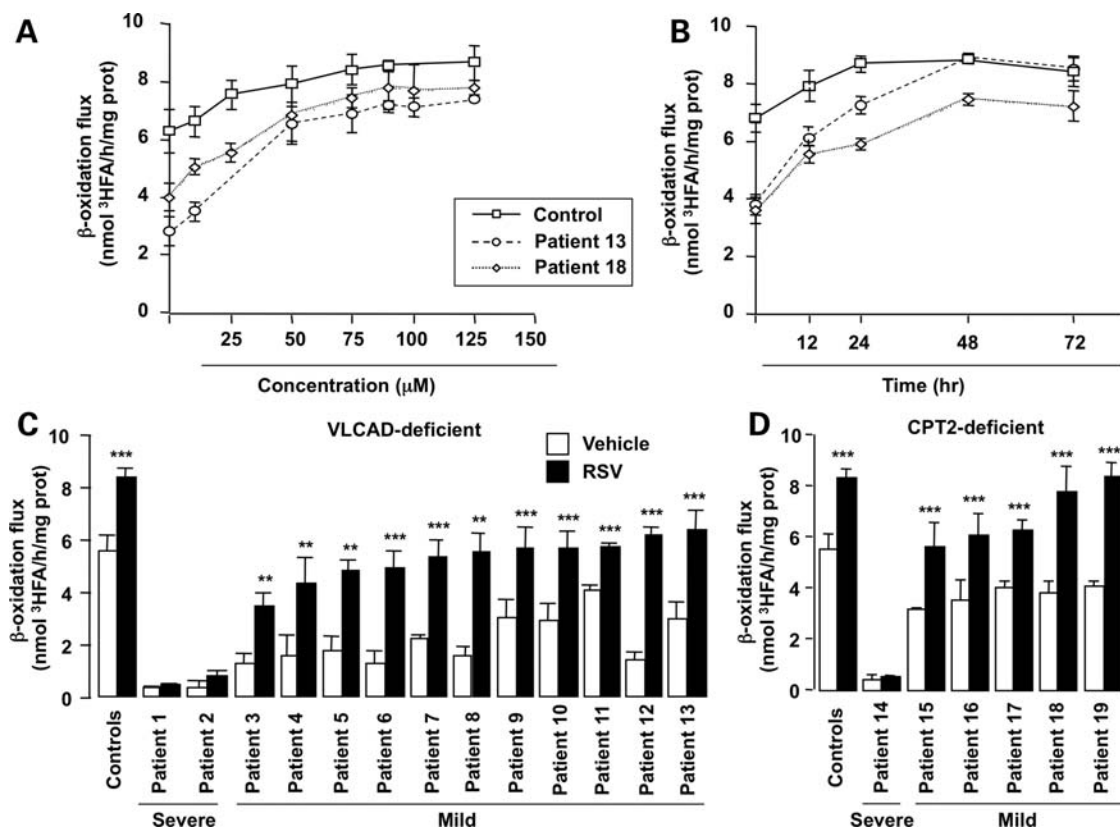


Figure 1. RSV increases palmitate oxidation in fibroblasts from control and FAO-deficient patients. (A) Dose-response studies of RSV effects on tritiated palmitate oxidation rates in control and VLCAD- or CPT2-deficient patients (Patient 13 and 18, respectively). The cells were treated for 48 h. (B) Time-course studies of RSV effects on tritiated palmitate oxidation rates in control and VLCAD- or CPT2-deficient patients. For the kinetics, the concentration of RSV used was 75 μM. For dose-response and time-course studies, results are means ± SD from two different experiments. In each experiment, the determinations were performed in triplicate. (C and D) β-Oxidation flux measured in fibroblasts of controls and VLCAD- or CPT2-deficient patients treated with 75 μM RSV or vehicle (DMSO) for 48 h. The results are means ± SD of at least three different experiments. In each experiment, the determinations were performed in triplicate. Control values are from three different healthy individuals. ***P* < 0.01; ****P* < 0.001 compared with vehicle-treated fibroblasts.

exhibited FAO rates ranging from 21% (Patients 3 and 6) up to 72% (Patient 11) of control values. Exposure to 75 μM RSV for 48 h induced a strong and significant increase of FAO capacities in control fibroblasts, and in the 11 cell lines with mild VLCAD deficiency. The increases of palmitate oxidation in RSV-treated fibroblasts varied from +50% (Patient 11) up to +360% (Patient 12) when compared with vehicle-treated cells (*P* < 0.001). Except for Patient 3, these inductions of FAO rates resulted in the restoration of normal palmitate oxidation in all the cell lines with mild VLCAD deficiency (Patients 4–13).

The effects of RSV were also tested in fibroblasts from the patients harboring different genotypes associated with the severe or with the mild form of CPT2 deficiency (Table 2). As shown in Figure 1D, fibroblasts from the severely affected Patient 14 exhibited a profound FAO deficiency, unchanged after treatment by RSV. In contrast, patients' fibroblasts with the mild form of CPT2 deficiency exhibited basal FAO rates ranging from 57% (Patient 15) to 72% (Patients 17 and 19) of control values. Furthermore, treatment with RSV markedly stimulated FAO utilization, resulting in a complete correction of FAO rates in the five cell lines with mild CPT2 deficiency.

RSV relieves the metabolic block by increasing the amount of mutated CPT2 or VLCAD protein

Fibroblasts from control, VLCAD- or CPT2-deficient patients were then treated or not with RSV (75 μM) for 48 h and submitted to western blot analysis. The amounts of VLCAD protein were significantly reduced in the fibroblasts, from –30% in Patient 10 up to –70% in Patient 4, compared with the normal level (Fig. 2A). In control and VLCAD-deficient patients, RSV clearly up-regulated the levels of VLCAD proteins to amounts similar to, and eventually higher than, those observed in untreated control cells (2- to 3.5-fold, *P* < 0.001). The same changes were observed in CPT2-deficient fibroblasts (Fig. 2B), which exhibited reduced amounts of CPT2 protein under basal condition (–21 to –63%), and a strong increase (2.5- to 4.6-fold, *P* < 0.001) in CPT2 protein levels in response to RSV treatment.

Real-time quantitative polymerase chain reaction (RT-PCR) was performed to determine the effects of RSV on mRNA levels of the VLCAD and CPT2 genes. As shown in Figure 2C and D, treatment with RSV resulted in a significant induction of VLCAD (+48 to +87%) and of CPT2 (+38 to +68%) gene expression in the control and the patients' cells.

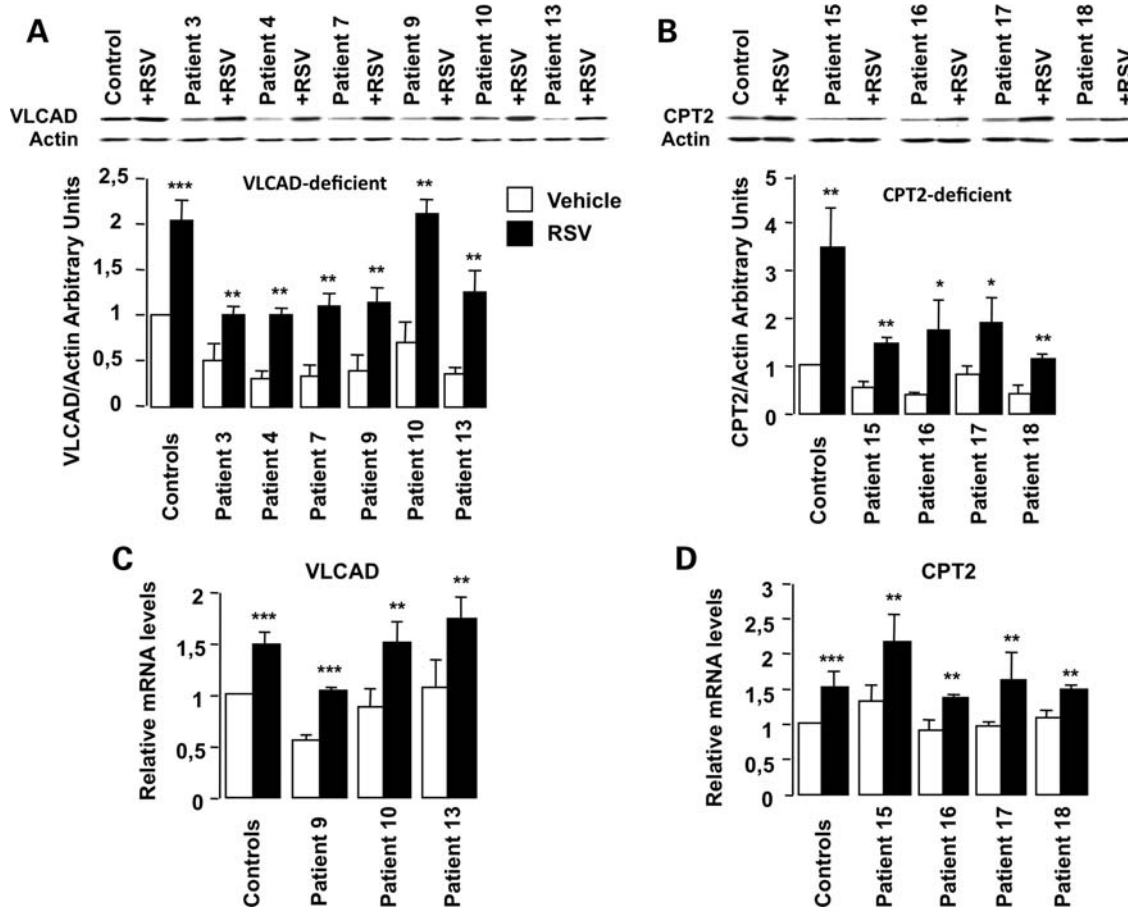


Figure 2. RSV increases VLCAD and CPT2 protein levels and gene expression. (**A** and **B**) Control, VLCAD- or CPT2-deficient fibroblasts were treated with 75 μ M RSV or vehicle (DMSO) for 48 h before protein extraction and western blot analysis. Histograms of protein amounts relative to β -actin are shown, as well as a representative western blot. Values are means \pm SD of three different western blots. * P < 0.05; ** P < 0.01; *** P < 0.001 when compared with vehicle-treated fibroblasts of a given individual. (**C** and **D**) Control, VLCAD- or CPT2-deficient fibroblasts were treated with 75 μ M RSV or vehicle (DMSO) for 48 h before ARN extraction and real-time quantitative RT-PCR analysis. In each experiment, quantitative RT-PCRs were run in triplicate. The results, expressed in arbitrary units, are means \pm SD of two different experiments. ** P < 0.01; *** P < 0.001 versus vehicle-treated fibroblasts.

Table 1. Mutations of VLCAD-deficient patients

Patients	Nucleotides change		Amino acid change		Phenotype
1	IVS8-2A>C	c.1322G>A	N252_H293del	G441D	S
2	c.553G>A	IVS8-2A>C	G185S	N252_H293del	S
3	c.520G>A	c.520G>A	V174M	V174M	M
4	c.1246G>A	c.1349G>A	A416T	R450H	M
5	c.848T>C	c.1322G>A	V283A	G441D	M
6	c.1144A>C	c.1339G>A	K382Q	G447R	M
7	c.790A>G	c.1309A>G	K264E	M437V	M
8	c.848T>C	c.1384G>A	V283A	E462K	M
9	c.664G>A	c.1512G>T	G222R	E504D	M
10	c.790A>G	c.790A>G	K264E	K264E	M
11	c.1367G>A	c.1844G>A	R456H	R615Q	M
12	c.997insT	c.1144A>C	A333fsX358	K382Q	M
13	c.848T>C	c.848T>C	V283A	V283A	M

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

The numbering of amino acids starts at the first methionine encoded by the translation initiation codon.

Phenotype at the time of diagnosis: S, severe; M, mild.

Correction of FAO by RSV requires SIRT1 and PGC-1 α

A number of biological effects of RSV have been proposed to involve SIRT1. This prompted us to investigate whether FAO

induction by RSV in human fibroblasts was dependent on SIRT1. We first verified that control and FAO-deficient fibroblasts expressed detectable levels of SIRT1 protein under standard culture conditions (Fig. 3B). Interestingly, it was found

Table 2. Mutations of CPT2-deficient patients

Patients	Nucleotides change		Amino acid change		Phenotype
14	c.983A>G	c.983A>G	D328G	D328G	S
15	c.338C>T	c.371G>A	S113L	R124Q	M
16	c.338C>T	c.112-113InsGC	S113L	S38Fs	M
17	c.338C>T	c.437A>C	S113L	N146T	M
18	c.338C>T	c.1883A>C	S113L	Y628S	M
19	c.338C>T	c.110-111dupGC	S113L	S38AFsX36	M

The numbering of nucleotides starts at the first adenine of the translation initiation codon.
The numbering of amino acids starts at the first methionine encoded by the translation initiation codon.
Phenotype at the time of diagnosis: S, severe; M, mild.

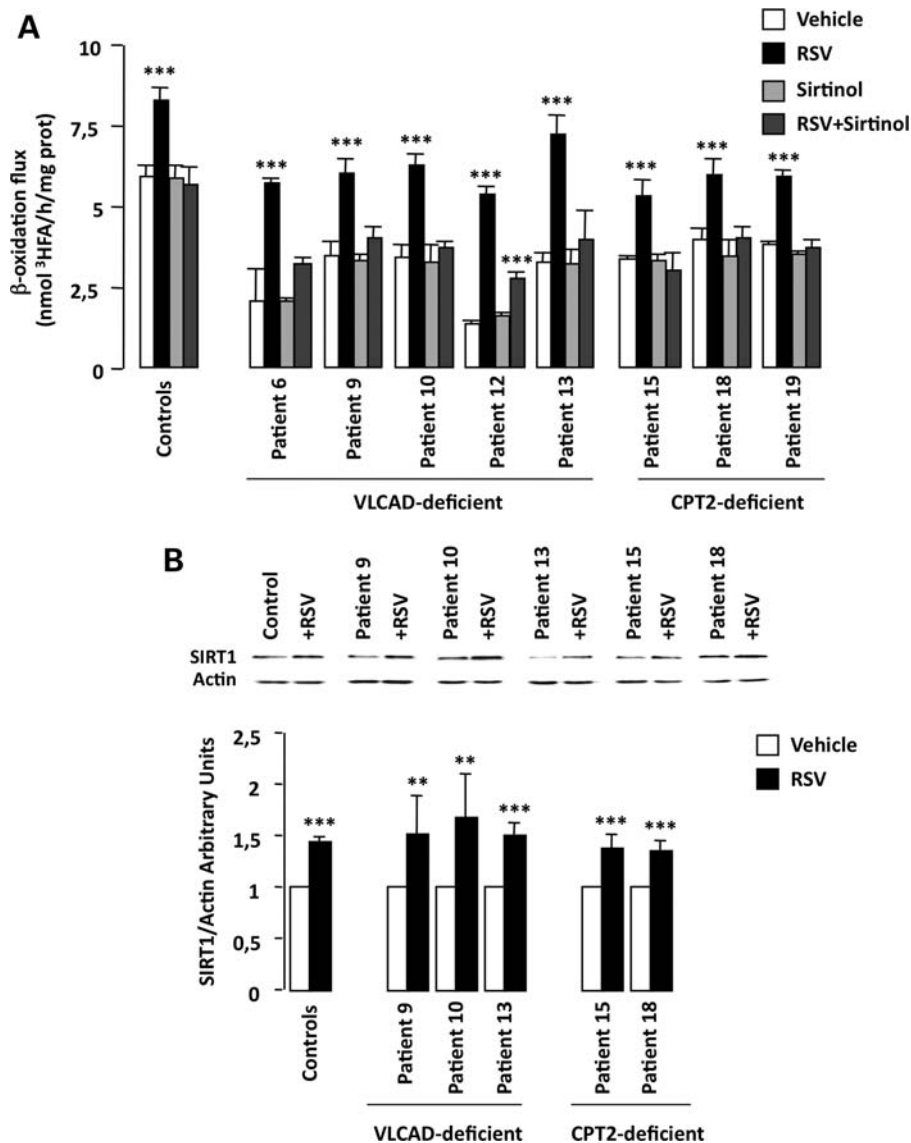


Figure 3. RSV action is mediated through SIRT1. **(A)** Fibroblasts of controls and VLCAD- or CPT2-deficient patients were treated with 75 μ M RSV, 40 μ M Sirtinol or 75 μ M RSV + 40 μ M Sirtinol or vehicle (DMSO) for 48 h. Palmitate oxidation rates were then measured. In each experiments ($n = 2$), the assays were performed in triplicate and the results are expressed as means \pm SD. *** $P < 0.001$ compared with vehicle-treated fibroblasts. **(B)** Cells were treated with 75 μ M RSV or vehicle for 48 h and SIRT1 protein levels were then determined by western blot analysis. Histograms of protein amounts relative to β -actin and a representative western blot are shown. Values are depicted as means \pm SD of three independent experiments. ** $P < 0.01$; *** $P < 0.001$ versus vehicle-treated fibroblasts.

that exposure to RSV increased SIRT1 mRNA (from +30 to +40%, data not shown) and protein levels (from +33 to +67%) (Fig. 3B) in control and in FAO-deficient fibroblasts. To address the possible role of SIRT1 in mediating the RSV effects, we studied the effects of Sirtinol, a known SIRT1 inhibitor. We used 40 μM of the compound in the culture medium, a concentration determined in preliminary dose-response experiments (data not shown). Control, VLCAD- and CPT2-deficient fibroblasts were treated with optimal doses of RSV, Sirtinol, or a combination of the two, for 48 h and then FAO flux was measured (Fig. 3A). Sirtinol alone had no significant effect on basal FAO rates. However, when tested in combination with RSV, Sirtinol appeared to fully suppress the FAO increases normally obtained in response to RSV. This inhibitory effect was observed in all the cell lines with the exception of Patient 12.

Because SIRT1 interacts with and activates PGC-1 α , and because PGC-1 α is a key co-activator of numerous genes involved in the FAO pathway, we investigated whether the effects of RSV on FAO were mediated by PGC-1 α in human fibroblasts. To this end, we performed siRNA experiments using control or PGC-1 α siRNA (Fig. 4A). The knock-down of PGC-1 α totally prevented the FAO increase that normally occurs in response to RSV treatment, in control as well as FAO-deficient patients.

We next determined the effect of treatment with RSV on PGC-1 α expression in control cells. PGC-1 α mRNA was significantly induced (50–60%) by RSV treatment (data not shown), and was associated with an increase (1.7-fold, $P < 0.001$) in PGC-1 α protein (Fig. 4B). We further determined, in parallel, whether PGC-1 α could be activated at the posttranslational level by deacetylation, in response to RSV. This was shown to be the case since immunoprecipitation experiments revealed that exposure to RSV decreased the acetylation state of PGC-1 α by 36%, consistent with its activation (Fig. 4B).

Activation of AMPK does not impact FAO in control or patient cells

Finally, since the effects of RSV could be mediated by AMPK, we asked the question as to whether the well-characterized AMPK activator, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), was able to induce FAO in human fibroblasts. As can be seen Figure 5A, 500 μM AICAR failed to increase the palmitate oxidation flux in control, VLCAD- or CPT2-deficient cells. Comparable results were obtained with 1 mM AICAR, whereas higher concentrations of this compound tended to inhibit FAO (data not shown). To confirm these results, we tested in parallel the effects of A-769662, a specific and direct activator of AMPK (28). A dose-response study was performed (Fig. 5B) which showed no significant changes in palmitate oxidation up to 100 μM . This dose was then tested in several other VLCAD- or CPT2-deficient patients, and it was found that A-769662 does not reproduce the effects of RSV on FAO in the various cells tested (Fig. 5C). Altogether, these data show that none of the two AMPK activators tested is able to induce a stimulation of FAO in the control or in the FAO-deficient human fibroblasts.

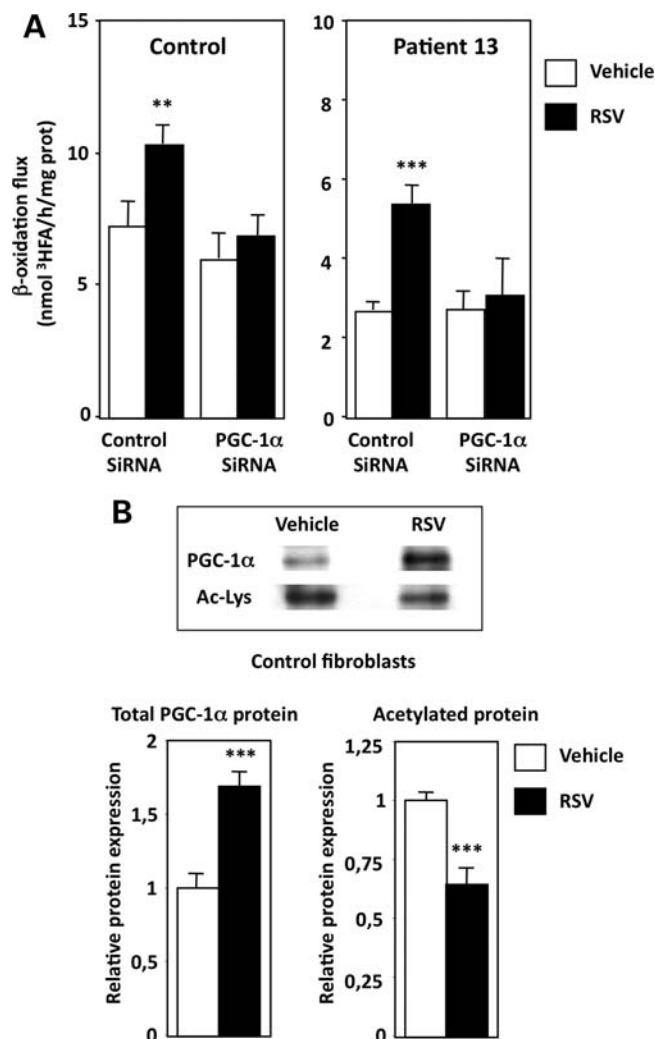


Figure 4. RSV action involves PGC-1 α . (A) Control or VLCAD-deficient fibroblasts were transfected with non-target (control siRNA) or PGC-1 α target siRNAs for 72 h before RSV treatment (75 μM , 48 h). Data are shown as means \pm SD of two different experiments. In each experiment, palmitate oxidation measurements were performed in triplicate. ** $P < 0.01$; *** $P < 0.001$ versus control siRNA-treated fibroblasts. (B) PGC-1 α was immunoprecipitated from nuclear extracts of control fibroblasts treated or not with RSV and western blot analysis was performed using PGC-1 α or acetyl-lysine antibodies. A representative western blot and quantifications are shown. Values represent means \pm SD of three independent experiments. *** $P < 0.001$ versus vehicle-treated fibroblasts.

DISCUSSION

RSV is a natural compound, produced by plants in response to bacterial or fungal attacks, which can be found in various food products such as red wine, grape juice or peanut butter. Besides its potential to inhibit carcinogenesis, or to protect against cardiac ischemic injuries (21), RSV has also been found to exert potent *in vivo* effects on energy and metabolic homeostasis. For example, it reduces fat deposits and improves insulin sensitivity in mice on a high-calorie diet (29,30). These metabolic effects target mitochondrial functions, since a global increase in mitochondrial oxidative metabolism and aerobic capacity has been reported in RSV-fed mice (29,30). However, the mechanisms have yet

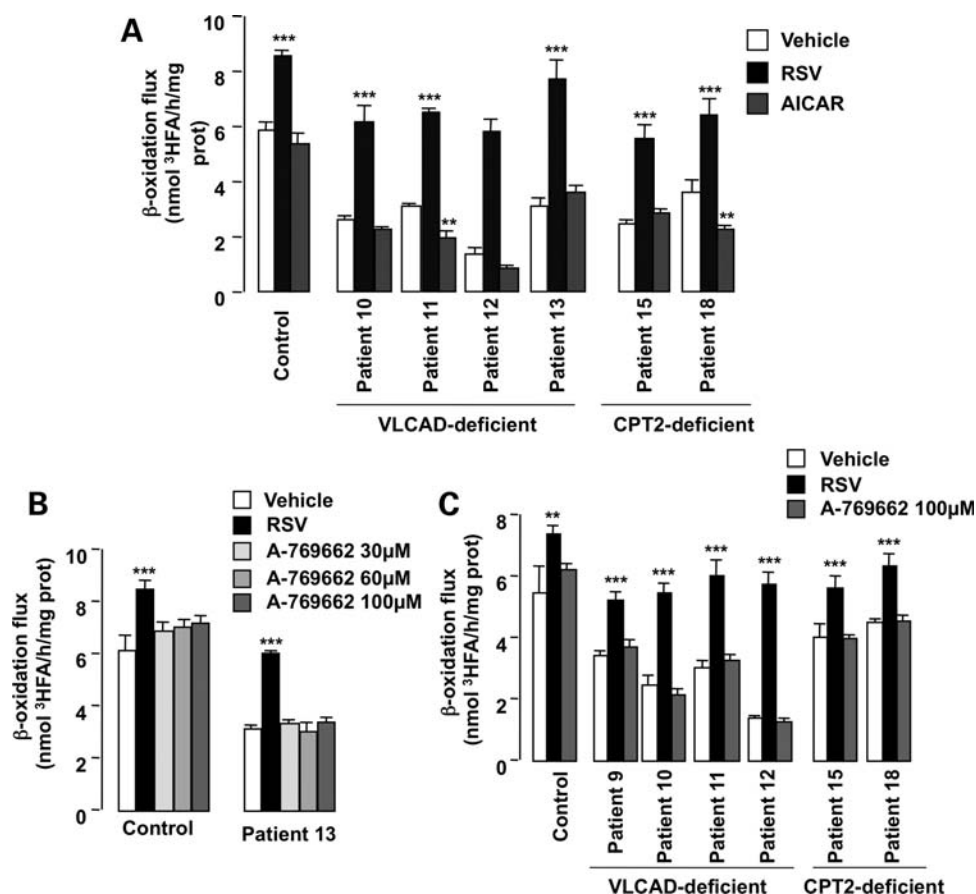


Figure 5. RSV action is not mediated through AMPK. (A–C) Control, VLCAD- or CPT2-deficient fibroblasts were treated with vehicle (DMSO) or 75 μ M RSV or 500 μ M AICAR, or different concentrations of A-769662 for 48 h before palmitate oxidation measurements. The results are means \pm SD of triplicates. Control values are from two different healthy individuals. ** $P < 0.01$; *** $P < 0.001$ compared with vehicle-treated fibroblasts.

to be documented. In the present study, we investigated the possible stimulatory effects of RSV on oxidative metabolism in human cells from FAO-deficient patients. To our knowledge, this is the first study that evaluates the potential of this compound in the field of inborn metabolic disorders.

Fibroblasts from patients with the myopathic form of VLCAD or CPT2 deficiency exhibited considerably lower VLCAD or CPT2 protein levels under basal conditions when compared with control fibroblasts. In the absence of RSV treatment, residual FAO capacities in the patients' cells varied from 20 to 70% of normal values. These features are typically associated with the presence of missense mutations which are characterized as mild, such as V174M (Patient 3), K264E (Patient 10) or V283A (Patient 5, 8 and 13) in the VLCAD gene (9), or S113L (Patients 15–19) in the CPT2 gene (6,14). Examination of these mutations in the crystal structures of the CPT2 or VLCAD proteins showed that none of them targets active site residues (31,32). Consistent with this, previous studies have established that there are variable amounts of low-residual VLCAD or CPT2 enzyme activities in cells from Patients 4, 5, 8, 9, 10, 13 or 15–17 (9,14). In contrast, cells from patients having the severe disease presentation harbor gene missense mutations which are in close vicinity to the enzyme active site (G185S, D328G), or which target highly conserved protein domains essential for catalysis (G441D), both of

which produce an extremely unstable protein (33) having negligible residual FAO capacities. The results obtained in this study provide evidence for a marked stimulatory effect of RSV on patient cell FA utilization. Indeed, exposure to RSV restores normal FAO capacities in a panel of cells harboring mild CPT2 or VLCAD deficiency. In all cases, correction of the FAO flux, in response to RSV, could be ascribed to a marked induction of mutated CPT2 or VLCAD mRNA and protein levels. These findings are consistent with literature data reporting an up-regulation of medium-chain Acyl-CoA dehydrogenase (MCAD) gene expression level in the muscle of mice chronically fed RSV (30). It should be noted that evidence for an RSV-induced stimulation of FAO have been, so far, based essentially on changes in the levels of β -oxidation gene expression. In this regard, our study identifies new RSV targets in the mitochondrial β -oxidation pathway, and it is the first to establish that exposure to RSV actually induces an increase in mitochondrial FAO flux in control human fibroblasts, and that exposure to RSV can restore normal FAO in genetically deficient cells from patients.

Our data suggest that these effects of RSV on mitochondrial β -oxidation involve SIRT1. This appears clearly from the experiments using Sirtinol, an SIRT1 inhibitor, since this compound fully abrogated the FAO stimulation normally observed in fibroblasts exposed to RSV. These results are in agreement

with data obtained in mouse embryonic fibroblasts (MEFs) from SIRT1^{-/-} mice, which showed that exposure to 50 μ M RSV increased MCAD mRNAs levels in SIRT1^{+/+} MEFs, but not in SIRT1^{-/-} MEFs, thus indicating a pivotal role of SIRT1 in mediating RSV effects on mitochondrial β -oxidation in mouse fibroblasts (30). *In vivo* studies in mice indicate that SIRT1 activation by RSV results in a coordinated regulation of SIRT1 downstream targets (29,30). Among these targets, PGC-1 α is considered to be a master regulator of mitochondrial energy metabolism and, in particular, of fatty acid β -oxidation (18). This led us to study the possible involvement of PGC-1 α in the fibroblast response to RSV. In our experiments, disruption of PGC-1 α expression by the use of PGC-1 α siRNA abolished the stimulation of FAO by RSV. Furthermore, the level of acetylated PGC-1 α was decreased in RSV-treated cells, consistent with an SIRT1-mediated activation of PGC-1 α in RSV-treated cells. RSV treatment also increased PGC-1 α mRNA and protein levels. Altogether, these observations clearly suggest that there is a global increase in PGC-1 α transcriptional activity, which could account for the up-regulation of the CPT2 and VLCAD genes in RSV-treated fibroblasts.

Interestingly, de-acetylation of PGC-1 α , and increased levels of PGC-1 α expression, has also been reported in the skeletal muscle of mice receiving chronic RSV supplementation (30). Furthermore, it was shown in the same study that up-regulation of MCAD gene expression in C2C12 muscle cells exposed to RSV was abolished by the use of PGC-1 α shRNA (30). Importantly, recent studies clearly indicate that RSV can also mediate significant *in vivo* effects over short periods of time. Thus, in high-fat fed mice, a 3-day treatment with SRT501 (a formulated RSV) led to an enhanced transcriptional activity of PGC-1 α and the up-regulation of FAO genes in the liver (34). A key role of the SIRT1–PGC-1 α axis in regulating FAO has also been confirmed by other groups, in C2C12 cells (35) and *in vivo*, by the use of liver-specific SIRT1 knockout mice (36,37). Interestingly, we found that RSV treatment led to an increase in the levels of SIRT1 mRNA and protein in human cells. This is in keeping with the recent observation that the human SIRT1 gene is under control of PPAR δ , a transcription factor known to be co-activated by PGC-1 α (38). Altogether, our results show that RSV-induced stimulation of FAO in normal or FAO-deficient human fibroblasts require the presence of PGC-1 α and SIRT1, in line with the results obtained in rodents.

In obese mice, the effect of RSV on energy metabolism has been proposed to be due partly to AMPK activation (29). RSV has also been reported to activate AMPK in neurons (25) and in HepG2 cells (26,27). It is admitted at present that AMPK can activate PGC-1 α by direct phosphorylation, thus leading to increase its transcriptional activity (39). It can thus be hypothesized that the effects of RSV observed in human fibroblasts might also involve AMPK. To address this issue, we evaluated the effects of two different AMPK activators: the well-known AICAR and the more specific AMPK activator A-769662 (28). Neither of these AMPK activators induced changes in the FAO capacities of control or deficient cells, suggesting that AMPK cannot mediate the induction of FAO by RSV in human fibroblasts.

In conclusion, this study demonstrates that RSV can exert potent regulatory effects on a key mitochondrial energy-

producing pathway in human cells. It also provides the proof-of-concept that these effects could be relevant for the pharmacological correction of inherited FAO deficiencies. In common metabolic disorders (obesity, type II diabetes), RSV could also be beneficial because of its potential to correct mitochondrial dysfunctions associated with these disorders (30). Other natural polyphenols, or so-called 'nutraceuticals', structurally related to RSV (butein, piceatannol, quercetin, etc.) have also been shown to activate SIRT1 (23) and could, therefore, elicit comparable metabolic effects, targeting mitochondrial oxidative metabolism. The involvement of SIRT1 in many biological processes, in particular ageing, has stimulated the quest for new SIRT1 activators and, recently, resulted in the identification of synthetic SIRT1 activators up to 1000-fold more potent than RSV (40). Among these, SRT1720 was shown to increase oxidative metabolism by stimulating fatty acid utilization in rodent cells (41). Other synthetic SIRT1 activators are also being tested in healthy volunteers or in patients with type II diabetes (<http://ClinicalTrials.gov>). Finally, clinical trials are in progress to test the effects of RSV in patients with colon cancer or Alzheimer disease, and to assess the safety, pharmacokinetics and pharmacodynamics of SRT501, a formulation of RSV with improved bioavailability (<http://ClinicalTrials.gov>). Obviously, the intriguing biological properties of RSV raise challenging questions concerning the functioning of key regulatory mechanisms in cell energy metabolism, and they have stimulated the research for treatments for a variety of common diseases. In the same line of reasoning, the effects of RSV on mitochondrial function might, hopefully, stimulate the research in the expanding area of human genetic disorders of mitochondrial origin, for which therapeutic approaches remain quite limited.

MATERIALS AND METHODS

Patients and control fibroblasts

The VLCAD- and CPT2- deficient human skin fibroblasts used for this study have been described previously (9,13,14), except Patient 19. Mutations, genotypes and phenotypes are given in Tables 1 and 2.

Cell culture and treatments

Fibroblasts 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 previously described (13). For treatment, the media were removed and the cells were incubated subsequently with fresh media containing the different effectors: 75 μ M RSV (Cayman, Ann Arbor, MI, USA) and/or 40 μ M Sirtinol (Sigma, St. Louis, MO, USA), or 500 μ M AICAR (Sigma), or 100 μ M A-769662 or with equivalent amounts of dimethyl sulfoxide (DMSO, vehicle). Cells were treated for 48 h.

Fatty acid oxidation measurement

Fatty acid oxidation was measured by quantitating the production of $^3\text{H}_2\text{O}$ from (9,10- ^3H) palmitate as described previously (14). For each cell line, FAO measurements were

performed in triplicate, and repeated in at least two independent experiments. The oxidation rates were expressed as nanomoles of ^3H fatty acid oxidized per hour per milligram of cell protein (nmol ^3H FA/h/mg protein).

Western blot analysis

Western blots were performed as described elsewhere (13). The following antibodies were used: anti-VLCAD (kindly provided by Dr S. Yamaguchi, Japan), anti-SIRT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti- β -actin (Chemicon International, Temecula, CA, USA). Immunoreactive bands were scanned by densitometry with a computerized video densitometer and the results were expressed as arbitrary units normalized to the amount of β -actin.

Real-time quantitative PCR

Total RNA from fibroblasts was isolated using Trizol reagent (Invitrogen), treated with DNase I (Ambion, Austin, TX, USA) and transcribed into cDNA as described previously (14). The real-time quantification of the different transcripts was performed using the SYBR Green kit from Thermo Fisher Scientific (Epsom, UK) and a LightCycler (Roche Diagnostics) according to the manufacturer's instructions. The primers for CPT2, VLCAD, β -actin and PGC-1 α have been published previously (13,42). The SIRT1 primers were: forward, GGGTACCGAGATAACCTTCTGT and reverse, TATCATC-CAACTCAGGTGGAGG. The results are expressed in arbitrary units normalized to the amount of β -actin.

siRNA experiments

Small interfering RNA (siRNA) targeting PGC-1 α sequences were synthesized by Sigma, as well as control siRNAs. siRNAs were transfected into fibroblasts using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

PGC-1 α acetylation assays

PGC-1 α lysine acetylation was analyzed by immunoprecipitation of PGC-1 α from fibroblast nuclear extracts followed by western blot. Fibroblast nuclear extracts were obtained using a kit from Pierce Biotechnology (Thermo Fisher Scientific, Epsom, UK) according to the manufacturer's instructions. PGC-1 α was immunoprecipitated using PGC-1 α antibodies (Santa Cruz Biotechnology). Total PGC-1 α protein levels and acetylated forms were detected using specific antibodies for PGC-1 α or acetyl lysine (Abcam, Cambridge, MA, USA).

Expression of results and statistical analysis

Data are the means \pm SD. Differences between groups were analyzed by one-way analysis of variance (ANOVA) and the Fisher test, or by the paired *t*-test; *P* < 0.05 was considered significant.

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Conflict of Interest statement. None declared.

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