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CPT1α over-expression increases long-chain fatty acid oxidation and reduces cell viability with incremental palmitic acid concentration in 293T cells ☆

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Abstract

To test the cellular response to an increased fatty acid oxidation, we generated a vector for an inducible expression of the rate-limiting enzyme carnitine palmitoyl-transferase 1α (CPT1α). Human embryonic 293T kidney cells were transiently transfected and expression of the *CPT1α* transgene in the tet-on vector was activated with doxycycline. Fatty acid oxidation was measured by determining the conversion of supplemented, synthetic *cis*-10-heptadecenoic acid (C17:1n-7) to C15:ln-7. *CPT1α* over-expression increased mitochondrial long-chain fatty acid oxidation about 6-fold. Addition of palmitic acid (PA) decreased viability of *CPT1α* over-expressing cells in a concentration-dependent manner. Both, PA and *CPT1α* over-expression increased cell death. Interestingly, PA reduced total cell number only in cells over-expressing *CPT1α*, suggesting an effect on cell proliferation that requires PA translocation across the mitochondrial inner membrane. This inducible expression system should be well suited to study the roles of CPT1 and fatty acid oxidation in lipotoxicity and metabolism in vivo.

Keywords: CPT1α; Long-chain fatty acids; Mitochondria; Lipotoxicity

Carnitine palmitoyltransferase 1 (CPT1) is one of the best regulated enzymes [1]. It catalyzes the transfer of long-chain fatty acids (LCFA) from CoA to carnitine for translocation across the mitochondrial inner membrane and is well recognized as the primary rate-controlling step in fatty acid oxidation [2,3]. Many studies [4,5] indeed indicate that the activity of CPT1 determines the rate of LCFA oxidation and there is one study reporting that over-expression of *CPT1α* in cultured β-cells increases fatty acid oxidation [6]. By regulating LCFA oxidation, CPT1 is also involved in other vital functions such as control of food intake [7–9], insulin secretion [6], and gluconeogenesis [10,11]. In most of these studies, CPT1 activity was manipulated pharmacologically [9,10] with substances whose actions were not wholly selective. An alternative approach is to modulate CPT1 expression by developing a transgenic animal model that permits controlled expression of *CPT1* in specific organs. Given the complex interactions and redundancies in physiological control systems, however, an acutely inducible over-expression of *CPT1* in specific

☆ *Abbreviations:* CPT, carnitine palmitoyltransferase; Dox, doxycycline; GFP, green fluorescent protein; LCFA, long-chain fatty acids; PA, palmitic acid; rtTA, Dox-inducible transactivator.

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tissues is presumably required to avoid the adaptive counterregulatory responses that would probably be provoked by constitutive transgene expression.

Modulation of CPT1 activity also may affect the toxicity of long-chain saturated fatty acids, such as palmitic acid (PA) [12–18]. Different groups have reported that pharmacological modulation that decreased [16–18] or that increased [12–15] CPT1 activity both enhanced PA-induced cell death and PA's antiproliferative effect. The reasons for such discrepant results are unclear. It is therefore important to examine whether transgenic *CPT1* over-expression affects cell viability.

This study had three aims. First, we wanted to develop a vector for a controlled expression of *CPT1α*—the liver isoform of CPT1. Second, we wanted to measure whether *CPT1α* over-expression increases fatty acid oxidation. Third, we wanted to measure the effect of *CPT1α* transgene expression on cell viability. Towards these aims, a tet-on gene expression vector with the rat *CPT1α* transgene [19] was generated and used to transfect human embryonic 293T kidney cells. To measure fatty acid oxidation, transfected cells were incubated with a fatty acid that does not occur naturally in mammals, C17:1n-7, and the unique oxidation product C15:1n-7 was measured. Finally, viability was measured in transfected cells incubated with different concentrations of PA.

Materials and methods

Cells and media. The cell culture media and reagents were purchased from Life Technologies (Basel, Switzerland) or Sigma (Buchs, Switzerland) unless otherwise indicated. The human embryonic kidney cell line 293T was chosen because these cells can be transfected very efficiently. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose. The medium was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin using a humidified incubator (37 °C and 5% CO_2). The cultures were split or used when they were approximately 80% confluent.

Plasmids. A tet-on gene expression system based on the *Escherichia coli* tetracycline-resistance operon was employed. The plasmid pUHrT62-1M2 [20] contains an improved doxycycline (Dox)-inducible transactivator (rtTA) under the control of the ubiquitously active CMV promoter. In the presence of Dox, the transactivator protein changes its configuration and can bind to a tetracycline-response element (TRE), where it acts as an enhancer. A second plasmid pUHD-CPT bears this TRE upstream of a minimal CMV promoter and the gene of interest, in this case *CPT1α*. The *CPT1α* cDNA was amplified by RT-PCR from rat liver poly(A^+) RNA and cloned downstream of the TRE-minimal CMV promoter. This construct permits a Dox-inducible expression of *CPT1α*. The cDNA insert was sequenced and compared with the rat mRNA sequence (NCBI database, U88294) [21]. The transfection efficiency was assessed with the plasmid pENFG1, a mammalian expression construct coding for enhanced green fluorescent protein (GFP) under the control of the CMV promoter.

Fatty acids. cis-10-Heptadecenoic acid (C17:1n-7) was bound to fatty acid-poor bovine serum albumin (20% w/v) at 37 °C overnight. This mixture was then added to the cell culture medium to give a final concentration of 0.25 mM C17:1n-7 and 2% BSA. PA (Fluka, Buchs, Switzerland) was dissolved in ethanol at 50 °C and then diluted with PBS to 20× final concentration. First, it was mixed with an equal volume of fatty acid-poor bovine serum albumin (20% w/v in PBS, BSA, Calbiochem, Switzerland; PBS, Gibco, Basel, Switzerland) at 37 °C for 30 min and then with the cell culture medium to give final concentrations of 1% ethanol, 1% BSA, and the PA concentrations described in the MTT assay.

RT-PCR analysis. 293T cells were seeded into 60 mm dishes at 1.5×10^6 cells per dish. At about 50–60% confluency the cells were transfected with either 4 μg pUHrT62-1M2 plus 4 μg pUHD-CPT or 4 μg pUHrT62-1M2 using PolyFect as a transfection reagent (Qiagen, Basel, Switzerland), according to the manufacturer's protocol. Non-transfected controls were treated without PolyFect DNA mix. Twenty-four hours after the transfection, the supernatant was removed and fresh cell culture medium containing 1 μg/ml Dox, 4 mM L-carnitine, and C17:1n-7 was added. After 24 h, the cells were washed, harvested, and counted.

Total RNA was isolated by adsorption to a silica gel membrane according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen). Total RNA concentration was measured with a photometer (at 260 and 280 nm). Possible contamination with genomic DNA was eliminated by digesting with RNase-free DNase (Protocol of DNase I, Invitrogen Life Technologies, Basel, Switzerland). Then two-step RT-PCRs with oligo(dT) primers and specific PCR-primers for human *CPT1α*, rat *CPT1α* or rtTA were performed. The PCR protocols were optimized to allow a semi-quantitative comparison of mRNA concentrations in differently transfected cells. The PCR products were analyzed on a 2% agarose gel and visualized with ethidium bromide.

Fatty acid oxidation assay. The transfection and incubation protocols were the same as above. Cells were seeded in 175 cm^3 flasks at 1.7×10^7 cells. The transfections were performed either with 18 μg pUHrT62-1M2 or with 18 μg pUHrT62-1M2 plus 18 μg pUHD-CPT. The cells were incubated with 0.25 mM C17:1n-7 for 24 h. The medium was removed and the cells were resuspended in methanol and stored at −70 °C. The cell suspensions were homogenized by sonication (Branson sonifier, Model 250, Branson Ultrasonics, Banbury, CT, USA; output 33 s), mixed with 2 ml transmethylation mix (methanol with 10% v/v concentrated sulfuric acid and 10% v/v hexane) and 20 μl internal standard (hexane with heptanoic acid, C7:0), and incubated at 50 °C overnight. The fatty acid methyl esters (FAME) were extracted with 1 ml hexane and 1 M NaCl ([22], method was modified for analysis). The FAME composition was analyzed using a gas-chromatograph (HP 6890, Hewlett-Packard, Philadelphia, Pennsylvania, USA) equipped with a Supelcowax-10 column (30 m × 0.32 mm, 0.25 m; Supelco, Bellefonte, USA). To calculate the relative amount of formed C15:1n-7, the ratio of the area under the C15:1n-7 gas-chromatograph curve to the area under the (C7:0) curve was divided by the total number of cells and multiplied by 10^7 cells. The identity of the target fatty acid (C15:1n-7) was verified with a mass spectrograph.

MTT assay. The MTT (3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, an index of cell viability and cell proliferation, is based on the ability of viable cells to reduce MTT from a yellow water soluble dye to a dark blue insoluble formazan compound [23]. 293T cells were seeded in 96-well tissue culture plates (15,000 cells/well). These were placed into an incubator for 24 h. The cells were then double transfected with 69 ng pUHrT62-1M2 and 69 ng pUHD-CPT. The control cells were also double transfected with 69 ng pUHrT62-1M2 and 69 ng pENFG1, and incubated for 48 h. The medium was changed and the cells were treated with various concentrations of PA for 18 or 24 h. For the last 2 h of treatment MTT was added to each well. The medium was aspirated and a freshly prepared lysis solution (5% formic acid in isopropanol) was added. The absorbance at 570 nm was determined with a multiwell plate reader (Dynatech, MR 5000, Bioconcept, Allschwill, Switzerland) with the background absorbance at 630 nm subtracted.

Trypan blue staining. To assess whether PA or *CPT1α* over-expression is cytotoxic, we stained the cells with trypan blue. 293T cells were transfected in the same way as for the MTT assay. The cells were incubated with 0.0 and 0.5 mM PA for 6 h, then harvested and stained with trypan blue (0.5%). The unstained (alive) and the blue cells (dead) were counted and the percentage of dead cells was calculated.

Statistical analysis. Data were analyzed with ANOVA (Statview, SAS Institute, Cary, NC, USA), with expressed gene (*CPT1α* or *GFP*) and PA concentration as independent factors. When significant differences were

found, post hoc analyses were performed with the sequentially rejective Bonferroni procedure [24]. The data on C15:ln-7 formation were evaluated with t tests. The differences were considered significant when $P < 0.05$.

Results

CPT1α expression and functionality

Human 293T cells co-transfected with rtTA and rat *CPT1α* vectors were tested for rat *CPT1α* expression. RT-PCR of double transfected cells revealed a band at 263 bp (Fig. 1A, lane 3), which is specific for rat *CPT1α*. This was not found in control groups transfected only with rtTA or without transfection (lanes 1 and 2). We also demonstrated that the transactivator was expressed in the single- and double-transfected cells, but as expected not in non-transfected cells (data not shown). Expression of the endogenous human *CPT1α* was detected in all three preparations (lanes 4–6). Although this technique does not permit an absolute quantification, there was clearly less human *CPT1α* mRNA in co-transfected cells (lane 6), probably due to down regulation of the endogenous *CPT1α* gene.

We then determined whether this over-expression of rat *CPT1α* in 293T leads to increased fatty acid oxidation. To avoid interference with the endogenous fatty acid metabolism, we added a synthetic fatty acid, C17:1n-7, that does not naturally exist in mammalian cells. Therefore, C15:ln-7 is the unique product of C17:1n-7 β-oxidation and C15:ln-7 concentration provides a measure of the cellular fatty acid oxidation rate. Double-transfected cells incubated with C17:ln-7 contained about 6-fold higher concentrations of C15:1n-7 than single or non-transfected cells (Fig. 1B). This result clearly shows that over-expression of *CPT1α* leads to increased mitochondrial β-oxidation.

Cell viability

After establishing that CPT1α indeed increases β-oxidation, we determined the cellular effects of this manipulation. Control experiments using a *GFP* expression vector showed that under these experimental conditions 70–80% of all 293T cells were transfected (data not shown).

After an incubation period of 18 h, cell viability was not reduced by PA in the control cells expressing *GFP*, but was reduced in a concentration-dependent manner at all PA concentrations tested in cells over-expressing *CPT1α* (significant interaction between gene [*CPT1α* and *GFP*] and PA concentration, ($F(1,496) = 11.69$, $P < 0.001$; Fig. 2A). The same result was obtained after 24 h for PA concentrations of 0.25 and 0.50 mM (significant interaction between gene [*CPT1α* and *GFP*] and PA concentration, ($F(1,496) = 8.319$, $P < 0.001$; Fig. 2B). We also assessed cell death because an increased rate of cell death and a reduced rate of cell proliferation can each reduce cell viability. *CPT1α* over-expression and 0.5 mM PA each increased cell death, as measured by trypan blue staining (main effects of PA, $F(1,1) = 22.01$, $P < 0.001$, and of gene, $F(1,76) = 4.12$, $P < 0.05$; interaction effect n.s.; Table 1). Interestingly, 0.5 mM PA decreased total cell number only in cells over-expressing *CPT1α* (interaction between gene and PA, ($F(1,76) = 4.80$, $P < 0.05$; Table 1).

Discussion

This study yielded several results towards the three aims defined in the introduction. First, we showed that human 293T cells transfected with a Dox-dependent transcriptional activator and the rat *CPT1α* gene under the control of a TRE-dependent promoter expressed the gene in the presence of Dox. This shows that the gene constructs with the Dox-dependent activation permit inducible

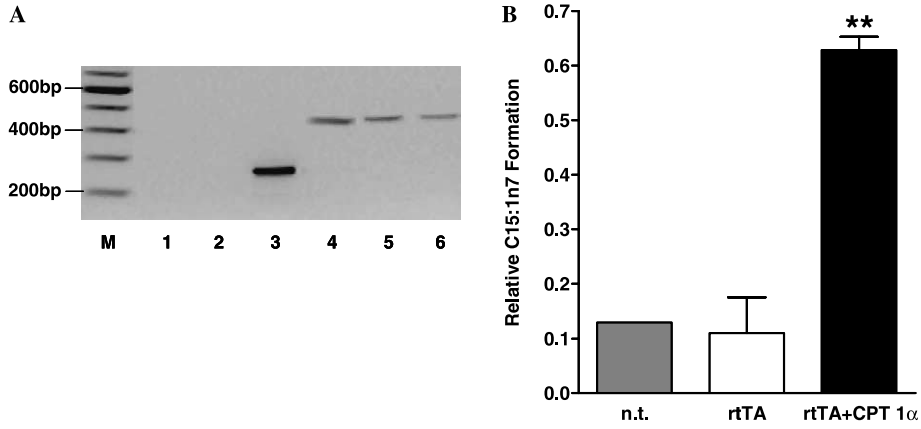

Fig. 1. *CPT1α* mRNA expression and β-oxidation rates in non-transfected, rtTA transfected, and rtTA plus *CPT1α* double-transfected 293T cells. (A) RT-PCR reveals a 263 bp band for transgenic rat *CPT1α* in double-transfected cells (lane 3), but not in non- (lane 1) or single- (lane 2) transfected cells. Endogenous human *CPT1α* could be detected in all three cell types (lanes 4–6). M: 100 bp molecular weight ladder. (B) β-oxidation rate as indicated by the relative oxidation of C17:1n-7 to C15:1n-7 in non-transfected (n.t.), single-transfected (rtTA), and double-transfected (rtTA + *CPT1α*) cells. Relative amounts of C15:1n-7 formation were calculated as described in Materials and methods. Data are means ± SEM; $n = 5$, except $n = 1$ for non-transfected cells. $^{**}P < 0.01$, rtTA vs. rtTA + *CPT1α*, t test.

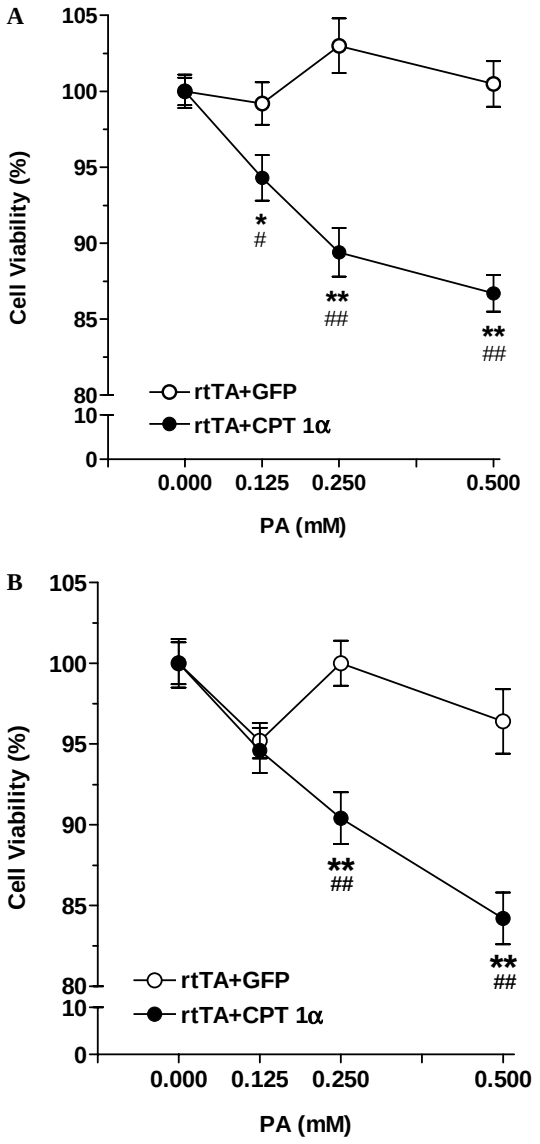

Fig. 2. Effects of *CPT1α* over-expression and palmitic acid (PA) on cell viability assessed by an MTT assay after incubation period of 18 h (A) or 24 h. (B) 293T cells were transiently transfected either with rtTA plus *GFP* (rtTA + *GFP*) or rtTA plus *CPT1α*(rtTA + *CPT1α*). Cell viability is expressed as percent of the 0 mM PA value. Data are means ± SEM; $n = 63$/group. $^{*}P < 0.01$, $^{**}P < 0.001$, vs. 0 mM PA in same genotype. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, rtTA + *CPT1α* vs. rtTA + *GFP*.

over-expression of *CPT1α* in living cells. Second, after incubation of transfected cells with the long-chain fatty acid C17:1n-7, an approximately 6-fold increase in C15:1n-7 (the first product of C17:1n-7 β-oxidation) was detected in double-transfected cells compared to single- or non-transfected cells. This demonstrates that over-expression of *CPT1α* in 293T cells increases fatty acid oxidation. The finding that transgenic manipulation of *CPT1α* can increase fatty acid oxidation in living cells encourages the use of these vectors in transgenic animals to test the roles of CPT1α activity and fatty acid oxidation in the control of certain metabolic functions. For example, this model should be useful in the studies of the effects of fatty acid oxidation in pancreatic β cells, in the liver, or in the brain on metabolism, food intake, and energy balance [6,8,10,11]. Inducible, organ-specific, expression of *CPT1* will also permit examination of the role of changes in fatty acid oxidation at different ages or in different metabolic situations in the absence of counter-regulatory responses caused by constitutive transgene expression.

Table 1
Palmitic acid (PA) and CPT1α over-expression on cell number and death assessed by trypan blue staining

	Cell death (%)	Total cell number
rtTA + GFP		
0.0 mM PA	4.2 ± 0.7	47663 ± 2379
0.5 mM PA[a]	7.9 ± 1.0	52713 ± 2723
rtTA + CPT1α[b]		
0.0 mM PA	5.7 ± 0.6	48775 ± 2594
0.5 mM PA[a]	9.7 ± 0.9	43088 ± 2052[c,d]

Data are means ± SEM, $n = 20$/group.
[a] Main effect of PA, $P < 0.001$.
[b] Main effect of gene, $P < 0.05$.
[c] 0.5 mM PA: rtTA + GFP vs. rtTA + CPT1α, $P < 0.05$.
[d] (0–0.5 mM PA) difference in rtTA + GFP vs. rtTA + CPT1α, $P < 0.05$.

We also demonstrated that *CPT1α* over-expression in 293T cells decreased cell viability with incremental PA concentrations, which is in agreement with some previous reports [12–14]. Reduced cell viability as assessed by an MTT test can be caused by reduced cell proliferation or increased cell death. Our trypan blue staining data indicated *CPT1α* over-expression indeed increased the percentage of dead cells, although this toxic effect was modest compared to the effect of increased PA concentration on cell death (i.e., *CPT1α* over-expression enhanced cell death by 20–40%, whereas incubation with PA increased the percentage of dead cells by 70–90% (Table 1)). More importantly, there was no interaction between the effects of *CPT1α* over-expression and PA, suggesting that the two induced cell death by separate mechanisms. Cell viability in *GFP* expressing cells, however, was not reduced by PA (Figs. 2A and B). This is apparently because in contrast to *CPT1α* over-expressing cells, *GFP* expressing cells compensated the increased cell death rate by a higher cell proliferation rate, as suggested by the higher number of *GFP* expressing cells than of *CPT1α* over-expressing cells after incubation with PA (Table 1). Others also have demonstrated effects of LCFA on cell proliferation [18,25,26]. In contrast to our findings, however, Berge et al. [18] demonstrated that in glioma cells inhibition rather than activation of CPT1α enhances the antiproliferative effect of PA. These results suggest that the physiological response to PA and CPT1α might be cell-type specific. The human cell line 293 is known for its good transfectability and high transgene expression [27], as apparently confirmed by our finding that *CPT1α* over-expression in these cells increased mitochondrial long-chain fatty acid oxidation 6-fold. The sensitivity of *CPT1α* over-expressing 293T human cells to LCFA-induced toxicity might therefore stem from the fact that these

are embryonic kidney cells that are not specialized for fatty acid oxidation. A comparison of different cell types might thus reveal interesting metabolic differences.

All in all, this study shows that the use of the tet-on expression vector can be used to induce expression of transgenic *CPT1α* and to increase cellular fatty acid oxidation in vitro. These findings provide a starting point for the use of transgenic over-expression of *CPT1* for in vivo tests of the various physiologic functions of fatty acid oxidation.

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