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PGC-1β regulates mouse carnitine–acylcarnitine translocase through estrogen-related receptor α

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ABSTRACT

Carnitine/acylcarnitine translocase (CACT) is a mitochondrial-membrane carrier proteins that mediates the transport of acylcarnitines into the mitochondrial matrix for their oxidation by the mitochondrial fatty acid-oxidation pathway. CACT deficiency causes a variety of pathological conditions, such as hypoketotic hypoglycemia, cardiac arrest, hepatomegaly, hepatic dysfunction and muscle weakness, and it can be fatal in newborns and infants. Here we report that expression of the *Cact* gene is induced in mouse skeletal muscle after 24 h of fasting. To gain insight into the control of *Cact* gene expression, we examine the transcriptional regulation of the mouse Cact gene. We show that the 5′-flanking region of this gene is transcriptionally active and contains a consensus sequence for the estrogen-related receptor (ERR), a member of the nuclear receptor family of transcription factors. This sequence binds ERRα *in vivo* and *in vitro* and is required for the activation of Cact expression by the peroxisome proliferator-activated receptor gamma coactivator (PGC)-1/ERR axis. We also demonstrate that XTC790, the inverse agonist of ERRα, specifically blocks *Cact* activation by PGC-1β in C2C12 cells.

1. Introduction

The carnitine/acylcarnitine carrier CACT transfers acylcarnitine esters, in exchange for free carnitine, across the inner mitochondrial membrane [1,2]. This transference is an essential step in long-chain fatty acid oxidation [3–5]. Mitochondrial oxidation of fatty acids provides the main source of energy during prolonged fasting as well as for skeletal muscle during exercise and for cardiac muscle. CACT deficiency is a rare autosomal recessive long-chain fatty acid oxidation disorder. Most patients become symptomatic in the neonatal period and show a rapid progressive deterioration and a high mortality rate [6].

As in other long-chain fatty acid disorders, the clinical management of CACT deficiency is limited and includes intravenous glucose (±insulin) administration to maximally inhibit lipolysis and subsequent fatty acid oxidation. A long-term strategy consists of avoidance of fasting, with frequent feeding and a special diet with restriction of long-chain fatty acids. In many cases the disease phenotype is correlated with residual cellular fatty acid oxidation capacities and it has been proposed that these disorders could be treated by increasing the expression of the mutant enzyme [7,8].

Homeostatic changes in gene expression in response to endogenous levels of small lipophilic molecules of hormonal, nutritional or metabolic origin are typically mediated by nuclear receptors. Orphan receptors are nuclear receptor-like molecules whose cognate lipophilic ligands and activation mechanism are unknown. ERRs were the first orphan receptors identified [9]. ERRs control large gene networks involved in energy homeostasis, including fat and glucose metabolism, as well as mitochondrial biogenesis and function [10]. ERR-mediated regulation of fatty acid β-oxidation genes is crucial in the control of energy balance in the body [11–15]. The transcriptional activity of ERRs is sensitive to the presence of coregulatory proteins that function as protein ligands for these receptors. Several studies have shown that ERRs mediate the activity of members of the family of PGC-1 co-activators; these molecules play essential roles in numerous processes, including mitochondrial biogenesis, adaptive thermogenesis, fatty acid β-oxidation and hepatic gluconeogenesis [16–20].

Little is known about the transcriptional regulation of *Cact* gene expression. Cact mRNA levels are induced in mouse liver after fasting [21]. Also, CACT concentration and carnitine transport are

Abbreviations: CACT, carnitine/acylcarnitine translocase; ERR, estrogen-related receptor; PGC-1, peroxisome proliferator-activated receptor gamma coactivator 1; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; ChIP, chromatin immunoprecipitation; CPT, carnitine palmitoyltransferase; CACL, carnitine/acylcarnitine translocase like; TR, thyroid hormone receptor.

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significantly lower in skeletal muscle mitochondria of insulin-resistant obese subjects than in healthy subjects [22]. Consistent with this observation, it has recently been described that the *Cact* gene is transcriptionally regulated in liver cells by PPAR through a functional and evolutionarily conserved PPRE [23,24].

Several lines of evidence point to the *CACT* gene as a target gene for ERR. The analysis of the effect of loss of ERRα function on global gene expression in the heart shows that, together with other genes involved in fatty acid metabolism, *Cact* is downregulated [12]. In SAOS2 cells, inhibition of ERRα expression by siRNA impairs the induction of mitochondrial biogenesis by PGC-1α and reduces the mRNA levels of genes up-regulated by PGC-1α, such as *CACT* [25].

Consistent with these results, here we show that the 5′-flanking region of the *Cact* gene contains a consensus sequence for ERR. This sequence binds ERRα both *in vivo* and *in vitro* and is required for the activation of Cact expression by the PGC-1/ERR axis. We also demonstrate that XTC790, an inverse agonist of ERRα, specifically blocks the activation of *Cact* by PGC-1β in C2C12 cells.

2. Material and methods

2.1. Cell culture and treatments

C2C12 myoblasts and Hek293A cells, obtained from the ATCC, were maintained in DMEM (Gibco) supplemented with fetal bovine serum (FBS) 10% (growth medium). For transient transfection assays, cells were typically transfected using Lipofectamine LTX reagent (Invitrogen).

2.2. Plasmids

pCact containing a 1037-bp fragment of the mouse Cact gene was constructed by PCR using the oligonucleotide primers DH1167: 5′-ACGCGTCAGTTCTCTGAGCTG CTGAG-3′ (MluI), and DH1168: 5′-CTCGAGACGACCTTTCACCTACATTC-3′ (XhoI), corresponding to coordinates −1110 to −1091 and −92 to −74 from the translation origin, respectively, and using Genomic DNA from C57BL6 mouse as template. The amplified product was purified and the MluI-XhoI-digested fragment was cloned into pGL3Basic (Promega). pmutCact containing point mutations at a putative ERRE sequence (−938/−930) was obtained by overlapping extension PCR, using the paired oligonucleotides DH1167: ACGCGTCAGTTCTCTGAG CTGCTGAG/DH1182 GAGAAAGTTAAaagCtTTGAGT AGAA, and DH1168: CTCGAGACGACCTTTCACCTACATTC/DH1181: TTCTACTC AaagCtTTTAACTTTCTC. pΔCact containing a 773-bp fragment of the mouse Cact gene was constructed by PCR using the oligonucleotide primers DH1180: 5′-ACGCGTTCCGATCTGAGATTGTTAGTCT-3′ (MluI), and DH1168: 5′-CTCGAGACGACCTTTCACCTACATTC-3′ (XhoI), corresponding to coordinates −846 to −825 and −92 to −74, respectively, and using pCact as template.

2.3. Transient transfection assays

Hek293A cells were transfected using Lipofectamine LTX reagent (Invitrogen) following the manufacturer's, instructions using 0.75 μg of reporter gene construct and the eukaryotic expression vectors: pCDNA3-ERRα (0.3 μg), pSV2-PGC-1α (0.6 μg), and pCDNA3-PGC-1β (0.6 μg), as indicated in the figure legends. We included 20 ng of plasmid pRL-CMV as an internal transfection control.

2.4. Firefly luciferase and Renilla luciferase assays

After 48 h, cells were harvested using the passive lysis method (Promega) (200 μL/well) and luciferase assays were performed with the Dual Luciferase Reporter Assay System (Promega), following the manufacturer's recommendations.

2.5. Adenovirus infection

C2C12 myoblasts were seeded at density of 3.5×10^5 cells in 60-mm dishes 24 h before infection. Media were then replaced by DMEM–FBS 2% (2 mL/60 mm-dish) and cells were infected with one of the following adenoviruses: AdCMV-mERRα (Vector BioLabs), AdCMV-mPGC1β (Dr. A. Vidal-Puig, University of Cambridge), or AdCMV-GFP (CBATEG). The multiplicity of infection of each adenovirus was 200. When indicated, cells were treated with XCT790 (1 μM) or vehicle (DMSO).

2.6. RNA isolation and real-time PCR analyses

Total RNA was extracted from isolated muscle tissues or C2C12 myoblasts with Tri-Reagent (Life Technologies). For quantitative real-time analysis of mRNA expression, TaqMan RT-PCR was performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems). Two micrograms of RNA was transcribed into cDNA using a M-MLV reverse transcriptase (Invitrogen) and random-hexamer primers (Roche Diagnostics). The reactions were performed in a final volume of 25 μL using TaqMan universal PCR master mix, No-AmpErase UNG reagent, and the specific gene expression primer pair probes. The Assay-on-Demand probes used were Mm00451571_m1 for mouse *Cact*, and eukaryotic 18S (Ref: 4319413E,) as an endogenous control (Applied Biosystems). The relative amount of *Cact* mRNA in each sample was normalized to a reference control using the comparative ($2^{-\Delta Ct}$) method and following the manufacturer's instructions.

2.7. Western blot assays

Protein extracts from C2C12 myoblasts were prepared by homogenization in a NP40 lysis buffer (NaCl 150 mM, Tris–HCl 50 mM, NP40 1%) supplemented with a cocktail of protease inhibitors (P8340, Sigma) and 0.1 mM phenylmethylsulfonyl fluoride. Proteins (20 μg/lane) were separated in an 8% SDS/PAGE gel, transferred to Immobilon-P membranes (Millipore, Bedford, MA) and probed with an antibody against ERRα (1:1000; PBS1x, Tween 1%, non-fat milk 5%) (sc-32972, Santa Cruz Biotechnology). The presence of ERRα was detected using a Donkey Anti-Goat IgG-Horseradish peroxidase conjugated (1:2000; PBS1x, Tween 1%, non-fat milk 5%) (sc-2020, Santa Cruz Biotechnology).

2.8. Electrophoretic mobility shift assay

ERRα was transcribed and translated *in vitro* using the expression plasmid pCDNA3-ERRα with the T7 TNT® Quick Coupled Transcription/Translation Systems (Promega). The EMSA assay was performed with the 2nd generation DIG Gel Shift Kit (03353591910, Roche), following the manufacturer's instructions. Briefly, two 38-bp probes were used. The wild-type probe included the putative binding site for ERRα: 5′-CCAACATTTCT ACTCAT GACCTTTAACTTTCTCCAGCC-3′ and the mutated probe had this region mutated as follows: 5′-CCAACATTTCTACTCAaagCtTTTAACT TTCTCCAGCC-3′.

2.9. Chromatin immunoprecipitation (ChIP) assay

Hek293A cells were transfected using Lipofectamine LTX reagent (Invitrogen), using 4.5 μg of reporter gene construct and the eukaryotic expression vectors pCDNA3-ERRα (1.8 μg) and pCDNA3-PGC-1β (3.6 μg). Fortyeight hours post-transfection, cells were treated with formaldehyde to a final concentration of 10%

and incubated at 37 °C for 10 min. The cross-link reaction was stopped by adding Glycine 0.125 M to the media and incubating cells for 5 min at room temperature. Cells were washed twice with PBS and 0.5 mM PMSF, scraped with 0.5 mL CEI collection (Tris–HCl 100 mM pH 9.4, DTT 10 mM and Protease inhibitor cocktail) The cell pellet was washed with PBS1x, resuspended with 1 mL NCPI buffer (EDTA 10 mM, EGTA 0.5 mM, Hepes 10 mM pH 6.5, TritonX-100 0.25% and Protease inhibitor cocktail) The pellet was then resuspended with 1 mL NCPII buffer (EDTA 1 mM, EGTA 0.5 mM, Hepes 10 mM pH 6.5, NaCl 200 mM and Protease inhibitor cocktail) Next, the pellet was lysed with 1 mL of Lysis Buffer (SDS 1%, EDTA 10 mM, Tris–HCl 50 mM pH 8, PMSF 1 mM and Protease inhibitor cocktail) for 10 min at room temperature. Lysated cells were sonicated in a Bioruptor™ Next Gen (Diagenode) to obtain 500–1000-bp fragments. The sonicated samples were centrifuged (10,000*g* for 10 min at 4 °C). Fifteem micrograms of chromatin per condition and antibody was diluted with IP buffer (Tris–HCl 20 mM pH 8, EDTA 2 mM, NaCl 150 mM, TritonX-100 1% and Protease inhibitor cocktail) to a final volume of 1 mL. The samples were pre-cleared for at least 2 h at 4 °C with unspecific IgG (10 μg) and Protein A/G PLUS-Agarose (20 μL) (sc-2003, Santa Cruz Biotechnology) blocked with salmon sperm DNA (0.5 μg/mL). They were then incubated for 16 h at 4 °C with 4 μg of the specific antibody (ERRα, ab16363: Anti-Estrogen Related Receptor α antibody - ChIP Grade) or rabbit IgG. During the last 4 h of incubation Magna ChIP™ Protein A + G (20 μL) (16–663, Millipore) was added to the samples. Magnetic beads were collected and washed for 5 min with WBI Buffer (Tris–HCl 20 mM pH 8, EDTA 2 mM, NaCl 150 mM, TritonX-100 1%, SDS 0.1%), WBII Buffer (NaCl 500 mM, TritonX-100 1%, SDS 0.1%), WBIII Buffer (Tris–HCl 10 mM pH 8, LiCl 0.25 M, NP40 1%, Sodium Deoxicholate 1%, EDTA 1 mM) and twice with TE buffer (Tris–HCl 10 mM pH 8, EDTA 1 mM). Precipitates were extracted twice with 200 μL of Elution Buffer (SDS 1%, $NaHCO_3$ 0.1 M) (5 min at room temperature) and the pooled eluates were heated at 65 °C for at least 4 h to reverse the cross-linking. The primers used for amplification were Luc For 5′-TAG-CAAAATAGGCTGTCCCCA GTG-3′ and DH1175 5′-ACTACTCATCAG GAGGAACTGT-3′. The PCR products were electrophoresed in a 2%

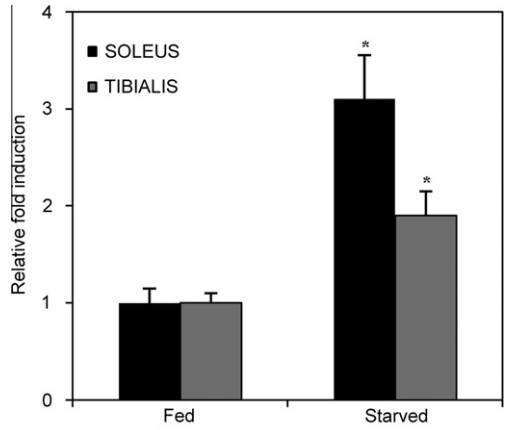

Fig. 1. *Cact* gene expression is induced in mouse skeletal muscle after 24 h of fasting. C57/BL6 male mice ($n = 5$) were fed *ad libitum* or fasted for 24 h, starting at the beginning of the dark phase of the light/dark daily cycle. Animals were killed and total RNA was obtained from *soleus* (black bars) and *tibialis anterior* (gray bars) muscles. *Cact* mRNA was analyzed by real-time PCR using 200 ng of retro-transcribed cDNA and TaqMan Cact-specific probes ($^{*}p < 0.05$ relative to animals fed *ad libitum*).

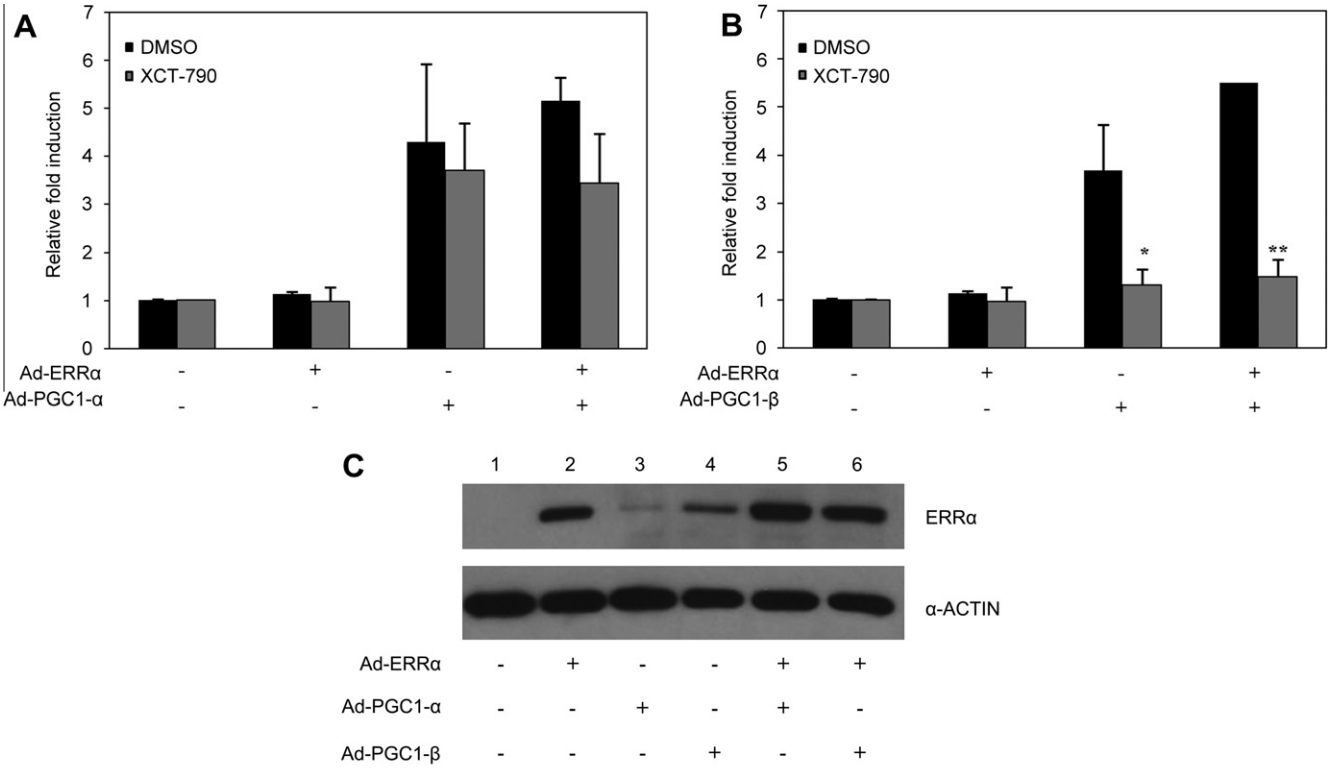

Fig. 2. *Cact* gene expression is induced by the PGC-1β/ERR axis in C2C12 cells. C2C12 myoblasts were transduced with recombinant adenoviruses expressing ERRα Moi 200, PGC-1α (Moi 200) (A) or PGC-1β (B), as indicated GFP was used as a control and to complete the adenovirus amount used in each condition. 32 h after the infection, an inverse agonist of ERRα, XCT790 (1 μM), or the vehicle (DMSO) was added to the culture media. 48 h after infection, RNA was obtained and *Cact* mRNA levels were quantified by real-time PCR using 200 ng of retro-transcribed cDNA and TaqMan *Cact*-specific probes ($^{*}p < 0.05$ an $^{**}p < 0.001$ relative to DMSO-treated cells). (C) 48 h after infection, 20 μg of protein extracts was used to analyze ERRα protein levels by Western blot. α-actin levels are shown as a loading control.

(w/v) agarose gel, visualized by staining with ethidium bromide, quantified by ImageJ and normalized by input amplification.

3. Results

3.1. Cact gene expression is induced in mouse skeletal muscle after 24 h of fasting

To study the effect of fasting on *Cact* gene expression in skeletal muscle, we applied real-time PCR to analyze *Cact* mRNA levels in *soleus* (predominantly oxidative) and *tibialis* anterior (glycolytic) muscles from 24-h fasted mice muscles from 24 h starved mice. *Cact* expression was significantly increased in both types of muscle (Fig. 1).

3.2. Cact gene expression is activated by the PGC-1β/ERR axis in C2C12 cells

Given the relevance of ERRα for adaptive energy metabolism, we checked the effect of this nuclear receptor and its co-activator PGC-1 on *Cact* mRNA levels. For this purpose, we infected C2C12 myoblasts with recombinant adenoviruses expressing PGC-1α or β and ERRα. *Cact* expression was not induced by ERRα alone but was activated by PGC-1α (Fig. 2a) or β (Fig. 2b), both in the presence or absence of ERRα. Given that PGC-1 elicits the expression of ERRα [19], we analyzed the effect of PGC-1 expression on endogenous ERRα levels by Western blot. The expression of either PGC-1α (lane 3) or β (lane 4) induced ERRα expression (Fig. 2c). To examine the participation of ERRα in PGC-1-induced *Cact* expression, we treated infected cells with XCT790, a selective ERRα inverse agonist. The agonist specifically blocked the effect of PGC-1β (Fig. 2b) but not α (Fig. 2a) on *Cact* gene expression.

3.3. The 5′-flanking region of the Cact gene contains a putative ERR binding site that binds ERRα both in vitro and in vivo

The *in silico* analysis of the 5′-flanking region of the *Cact* gene with PROMO, a software package that searches for putative transcription binding sites [26], showed the presence of a putative ERR binding site, TGACCTTTA, 808 bp upstream of the transcription start site of the gene. This sequence bound ERRα both *in vivo* and *in vitro*, as shown by Gel shift experiments (Fig. 3a) and ChIP (Fig. 3b). The latter experiments also revealed that PGC-1β increased ERRα binding to the response element located in the *Cact* promoter.

3.4. Cact gene transcription is under the control of the PGC-1/ERR axis

In order to check the functionality of this sequence, we cloned a fragment of the 5′-flanking region of the *Cact* gene, from −980 to + 55 relative to the transcription start site driving the activity of a luciferase reporter gene. As shown in Fig. 4 (minigraphs), this region contains a functional promoter that drives the transcription of the luciferase reporter in Hek293A cells. This figure also shows the transcriptional activity of two constructs containing a mutated version of the putative ERR responsive element (pmutCact) or a deletion of the 5′-flanking region of the *Cact* gene that does not carry this element (pΔCact). Neither deletion nor mutation of this

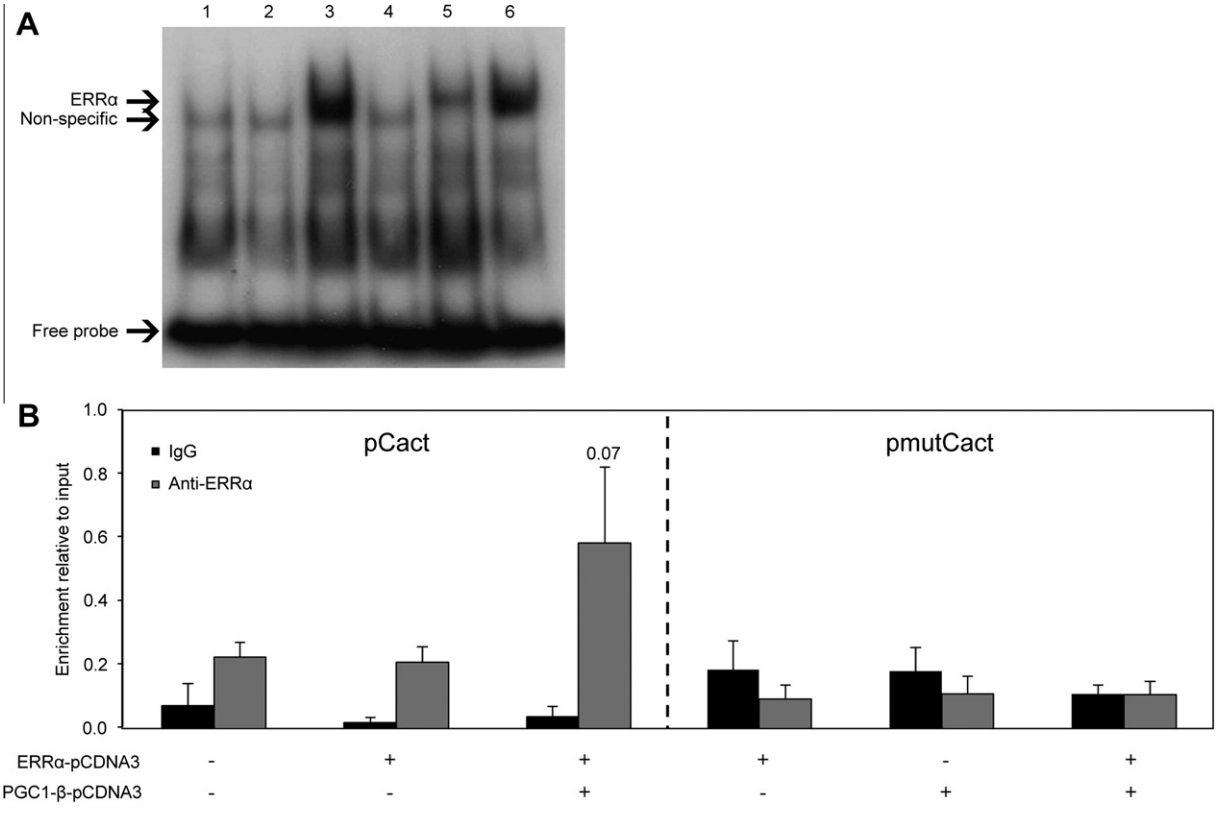

Fig. 3. A putative ERR binding site in the 5'-flanking region of the *Cact* gene binds ERRα *in vitro*. (A) Gel mobility assays were performed using the putative binding sequence described on the mouse *Cact* promoter as a probe. Binding of ERRα to this site was confirmed using ERRα protein produced by TnT. The protein interacted specifically with the wild-type probe (lane 3) but not with the mutated one (lane 4). The protein-DNA interaction was reversed with an excess of cold wild-type probe (lane 5) but not with cold mutated probe (lane 6). (B) Chromatin immunoprecipitation assays were performed with chromatin from Hek293A cells transfected with 4.5 μg of reporter gene construct pCact or pmutCact and the expression vectors pCDNA3-ERRα (1.8 μg) and pCDNA3-PGC-1β (3.6 μg), as indicated. The chromatin obtained was immunoprecipitated with rabbit IgG (black bars) or with an ERRα-specific antibody (gray bars). The results were normalized by inputs.

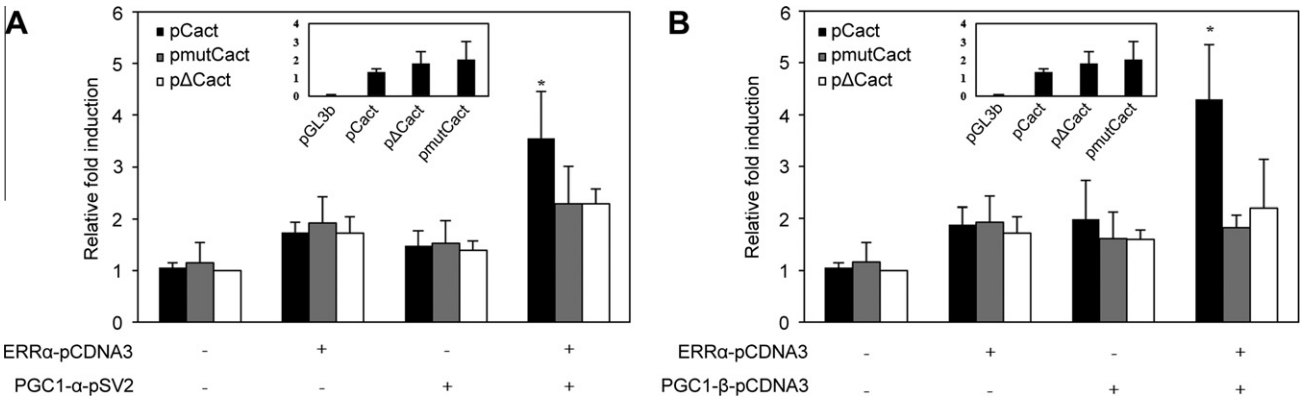

Fig. 4. *Cact* gene transcription is under the control of the PGC-1β/ERRα axis. Hek293A cells were cotransfected with the luciferase reporter constructs pCact, pΔCact or pmutCact (0.75 μg each) and the expression plasmids for ERRα 0.3 μg), PGC-1α 0.6 μg) (A), and PGC-1β 0.6 μg) (B), as indicated. Forty eight hours after transfection, cells were harvested and luciferase activity was measured. Results show *Renilla*-normalized luciferase activity. (Minigraph)- To check the basal activity of the promoter constructs, Hek293A cells were transfected with the luciferase reporter constructs pGL3b, pCact, pΔCact or pmutCact alone. Forty eight hours after transfection cells were harvested and luciferase activity was measured. Results show *Renilla*-normalized luciferase activity (mean ± SD from four independent experiments done in duplicate). (*$p < 0.05$ relative to basal activity).

sequence altered the basal transcriptional activity of the *Cact* gene promoter.

To check the effect of ERRα and PGC-1 isoforms on the transcriptional activity of the *Cact* gene, we transfected Hek293A cells with various reporter constructs and a combination of expression plasmids for ERRα and PGC-1α or β. Maximal activity of the reporter was observed in the presence of both ERRα and PGC-1α or β and mutation or deletion of the ERRα binding site significantly reduced the response of the reporter to ERRα in the presence of either PGC-lα or β (Fig. 4a and b). These findings indicate that this sequence is a genuine ERRα responsive element.

4. Discussion

Against the well-established idea that expression of the CPT system (except the *CPT1A* gene) is constitutive, several observations show that *CPT1A*, *CPT2* and *CACT* genes are also induced in situations of high demand for fatty acid oxidation such as fasting. The *Cact* gene, for instance, is induced during fasting in mouse liver [21]. We determined the muscle mRNA levels of *Cact* in mice after 24 h of fasting using real-time PCR and found a significant increase both in soleus and in tibialis anterior. The increase was higher in the former, which is consistent with the abundance of type I oxidative fibers in this muscle and the oxidation of fatty acid as the energy source.

The control of energy homeostasis is achieved by the coordinated action of several transcription factors and associated coregulators. The nuclear receptor family of transcription factors plays an important role in this metabolic regulation of transcription because they translate hormonal, nutrient and metabolic signals into specific gene expression networks to satisfy energy demands in response to physiological conditions. In this context, ERRs are orphan nuclear receptors whose transcriptional activity depends on the presence of coregulatory proteins, particularly the members of the family of PGC-1 co-activators, which act as protein ligands for ERRs and play a crucial role in the control of energy homeostasis. Several lines of evidence show that the *CACT* gene is under the control of the PGC-1/ERR axis; however, to date, *CACT* has not been identified as a direct target of ERRs. ChIP-on-Chip data showed that the homologous protein-coding gene *CACL* (CACT-like), is occupied by ERR gamma in mouse adult and neonatal heart [12,27]. Here we demonstrate that the infection of C2C12 cells with recombinant adenoviruses for PGC-1α or β results in the induction of *Cact* mRNA. We also show that the response to PGC-1β but not α is ERR-dependent, since although both PGC-1 isoforms elicited the expression of CACT and ERRα, only the activation produced by PGC-1β was affected by XCT790, an inverse agonist of ERRα. These results coincide with the idea that PGC-1β is a key regulator of ERR-mediated transcription in the control of energy expenditure; a notion supported by *in vivo* studies using transgenic mice overexpressing PGC-1β [20]. The activation of *Cact* expression in C2C12 in response to PGC-1α in an ERRα-independent way may be attributable to the potential of PGC-1α, unlike PGC-1β, to activate many nuclear receptors.

The ERRα-mediated induction of the *Cact* gene could be a direct effect of this gene or the result of the activation of other transcription factors such as PPARα [15], or TRα1 [28]. In this study, we localized a functional ERR binding site starting 808 bp upstream of the mouse *Cact* gene transcription start site. This sequence bound ERRα *in vitro*, as shown by gel shift experiments, and *in vivo*, as demonstrated by ChIP. Also, mutation or elimination of this sequence diminished the activation of *Cact* promoter reporter constructs triggered by PGC-1α and PGC1β and ERRα. These results identify the *Cact* gene as a direct target of ERRα co-activated by PGC-1β.

CACT deficiency is a life-threatening disorder; the prognosis of the disease depends on genotype, early diagnosis and prompt treatment. Our observations contribute to elucidating the mechanisms that regulate the expression of CACT and consequently the functionality of the CPT system, the main regulatory step of mitochondrial fatty acid β-oxidation. Our results also further our understanding of the effects of ERRα and PGC1β in the network of signals that control lipid metabolism, adding *Cact* as a genuine gene target of these transcriptional regulators.

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