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CHARACTERIZATION OF A RESPONSE ELEMENT FOR PEROXISOMAL PROLIFERATOR ACTIVATED RECEPTOR (PPRE) IN HUMAN MUSCLE-TYPE CARNITINE PALMITOYLTRANSFERASE I

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1. INTRODUCTION

Metabolic control of gene transcription in bacteria or yeast is a well-documented issue since the pioneer studies of gene expression. Recent observations indicate that transcriptional control of gene expression by metabolites can also be functional in mammals.[1–4] The identification of mammalian nuclear receptors for fatty acids and eicosanoids (PPAR) is of relevance because it establishes a link between the metabolism of fatty acids and transcriptional control, suggesting a molecular mechanism by which dietary fatty acids can modulate lipid homeostasis. The fact that fatty acids are ligands of PPAR,[5,6] a nuclear receptor that induces gene transcription of a suit of genes involved in lipid metabolism, indicates that fatty acids and related molecules can regulate the metabolism by short- (allostreric control) and long-term (transcriptional control) mechanisms.

We have identified the mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase gene as a PPAR target, and we have found that this receptor, which mediates the induction of the gene by fatty acids, binds as a PPAR-RXR heterodimer to a

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response element for peroxisomal proliferator activated receptor (PPRE) in the promoter region of the rat and pig genes.[7,8] Rat mitochondrial HMG-CoA synthase PPRE is in fact a nuclear receptor-responsive element (NRRE) since, in addition to **RXRα-PPAR** heterodimer, other nuclear receptors, like chicken ovalbumin upstream-promoter transcription factor (COUP-TF) or hepatocyte nuclear factor 4 (HNF-4) it bind to the element.[9,10]

Other genes involved in mitochondrial lipid metabolism, such as acyl-CoA synthetase[11] or medium-chain acyl-CoA dehydrogenase (MCAD),[12] are also targets of PPAR, indicating that different steps of fatty acid metabolism, like activation, oxidation, and utilization of fatty acids are regulated by the levels of the substrate, the fatty acids. Therefore, we speculated that the main control step in fatty acid **β-**oxidation, the outer membrane component of carnitine palmitoyltransferase enzyme system, CPT I, could also be a PPAR target.

Two isoforms of CPT I have been described, which have been designated LCPT I and MCPT I, since these isoforms are mainly expressed in liver and muscle respectively. The MCPT I gene is expressed not only in skeletal muscle but also in heart and brown and white adipose tissue.[13] This expression pattern may be of great significance since fatty acids are a major source of energy for heart, skeletal muscle and brown adipose tissue. We have amplified by polymerase chain reaction (PCR) the 5′ region of the human heart and brown adipose tissue CPT I gene[14] and demonstrated, first, the transcriptional activity of this fragment and second, the presence of a PPRE in the 5′-flanking region of this gene. In CV1 cells, the activation of the CPT I gene by PPAR was dependent on the addition of exogenous ligands.

2. RESULTS AND DISCUSSION

2.1. Fatty Acids Regulate the Mitochondrial Synthase Gene Expression Mediated by PPAR: Localization of the PPRE

There is considerable evidence that fatty acids can activate PPAR as potently as peroxisome proliferators do.[15–17] On the other hand, previous results in our laboratory revealed that fatty acids induce an increase in the mitochondrial HMG-CoA synthase mRNA levels[18] and gene transcription.[19] We were therefore interested in determining whether fatty acids could have a role in the regulation of the mitochondrial HMG-CoA synthase gene expression mediated by PPAR. Figure 1 shows that the highest stimulation of the mitochondrial synthase-CAT reporter plasmid was observed after the induction with fatty acids in the presence of PPAR. In agreement with previous results[16,17] we observed that monounsaturated oleic acid is less potent than linoleic acid in the activation of PPAR.

Experiments of 5′nucleotides deletion, electrophoretic mobility shift assays (EMSA) and site-directed mutagenesis localized a PPRE in the mitochondrial HMG-CoA synthase promoter (between positions –104 and –85),[7] which is composed, like other PPREs,[20] of an imperfect direct repeat of the consensus binding sequence for the nuclear receptor superfamily with a spacing of a single base pair (DR-1), and a conserved sequence flanking the second repeat of DR-1. Figure 2 A shows the localization of putatives PPREs in the mitochondial HMG-CoA synthase gene of three different species.

Besides the characterization of mitochondrial HMG-CoA synthase as a PPAR

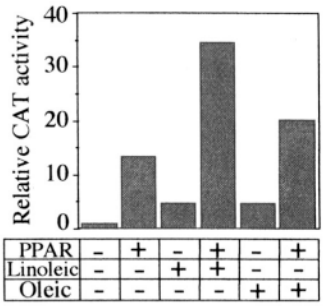

Figure 1. *Transcriptional activation of the rat mitochondrial HMG-CoA synthase gene promoter by fatty acids.* HeLa cells were cotransfected with 100 ng of the expression vector for mPPARα and reported plasmid pSMPCAT1 (−1148 to +28) in the presence or absence of 150 mM linoleic acid or 250 mM oleic acid as indicated at the bottom of the figure. Average value of β-galactosidase-normalized CAT activity are expressed as relative CAT activity, with the activity in the absence of both PPAR and fatty acids defined as 1.

target gene we had two more objectives: i) to identify other nuclear receptors able to bind to the mitochondrial HMG-CoA synthase PPRE, and ii) to extend our previous observation on mitochondrial HMG-CoA synthase gene to other genes related with fatty acids metabolism.

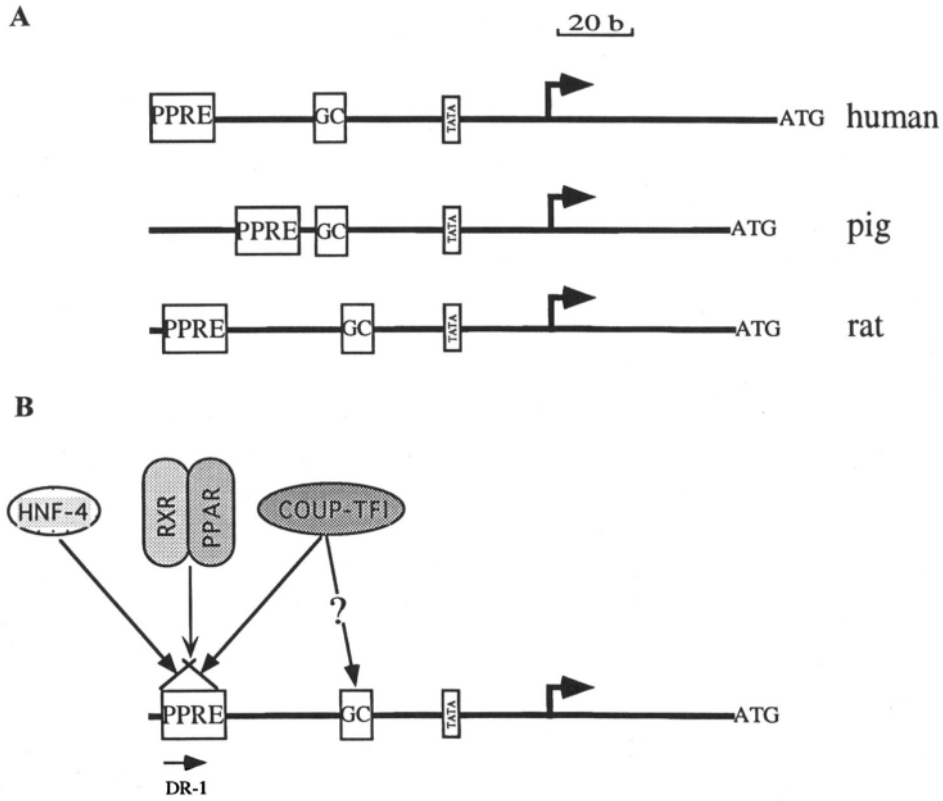

Figure 2. *Up-stream elements (UPEs) organization of human, pig and rat mitochondrial HMG-CoA synthase genes.* (A) The transcription start site, indicated by arrows, was determined by 5-RACE for the human[25] and pig[26] genes, and by primer extension and S1 experiments for the rat [27] gene. The functionality of PPRE was showed experimentally for the rat[7] and pig gene.[8] Human PPRE showed 17/19 identity with rat PPRE.[25] The scale at the top is in nucleotides. (B) Rat mitochondrial HMG-CoA synthase PPRE is a NRRE. Proposed model for the action of COUP-TF I[9] and HNF-4[10] on the rat gene.

2.2. COUP-TF I and HNF-4 Binds to a Nuclear Receptor-Responsive Element (NRRE) in the Mitochondrial HMG-CoA Synthase Gene

Rat PPRE of mitochondrial HMG-CoA synthase is a nuclear receptor responsive element (NRRE). We have shown (see Fig. 2 B) that despite the binding of PPAR/RXR heterodimers to the PPRE, which transactivates the promoter activity, COUP-TF can bind as a homodimer to rat PPRE, competing with the PPAR-RXR binding and therefore abolishing the PPAR transactivation.[9] We also showed that COUP-TF can form non-functional heterodimers with PPAR or RXR yielding the same effect: the lack of transactivation in the presence of PPAR.[9] HNF-4 is another nuclear receptor that can bind to rat PPRE. This binding does not require the complete element, only the DR-1 of the element, and it produces a similar effect in transcription activity to COUP-TF.[10] Besides the binding to a PPRE just described, COUP-TF can trans-activate promoter constructs that carry only the GC and TATA box. Moreover, the effect of COUP-TF on this small piece of DNA is tissue-[9] and species-specific (Judith Mallolas, and Pedro F. Marrero, unpublished). These results suggest that the mitochondrial HMG-CoA synthase gene is subjet to different regulation by the interplay of multiple members of the nuclear hormone receptor superfamily.

2.3. Human MCPT I gene 5′ Flanking Region Contains a Consensus PPRE and It is Activated by PPAR

In addition to HMG-CoA synthase, involved in ketogenesis, genes involved in fatty acid activation, like acyl-CoA synthetase,[11] or in β-oxidation, like medium-chain acyl-CoA dehydrogenase[12] are also target of PPAR. This transactivation pathway is reminiscent of a prokaryotic operon organization, in which fatty acids induce the expression of the genes responsible for their metabolism. We speculated that the main control step in fatty acid β-oxidation, the outer membrane component of carnitine palmitoyltransferase enzyme system, CPT I, could also be a PPAR target.

The 5′ flanking regions of CPT genes is now known.[14,21–23] Human Muscle type CPT I had been cloned,[14] and the complete sequence of the human gene from a BAC clone containing the end of the q arm of chromosome 22 is in GenBankTM (U62317). The analysis of the 5′-flanking region of this gene by TFSEARCH routine, performed using the Kyoto center's GenomeNet WWW Server, shows the presence of a putative PPAR binding sequence upstream of exon 1A (see Fig. 3A). The comparison of this sequence with the consensus sequence required for the binding of the PPAR-RXR heterodimer, as proposed by Palmer *et al.*,[20] shows the coincidence of 17 out of 20 bases (see Fig. 3B).

We performed gel mobility shift assays to analyze whether PPAR-RXR heterodimers bind to the putative PPAR binding sequence of the human muscle type CPT I gene. As can be seen in Fig. 4A neither PPARs nor RXR alone binds significantly to this sequence. However, incubation of this probe with a mixture of PPAR (α, β or γ) and RXRα resulted in a prominent complex. An oligonucleotide containing a mutated PPRE was not able to compete with the wild type probe for the formation of the complex (see Fig. 4B). The binding of the three subtypes of PPAR to the MCPT I PPRE is as strong as the binding to the mitochondrial HMG-CoA synthase PPRE, which allows the formation of the strongest complexes for all PPAR subtypes[24] (data not shown).

To investigate the effect of the observed binding of PPAR to the human MCPT I gene promoter on its transcriptional activity, we made constructs in which

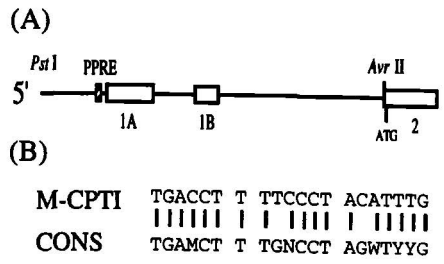

Figure 3. *Scheme of the 5′-flanking region of the human MCPT I.* (A) The positions of exons 1A, 1B and 2 containing the translation initiation codon (open rectangles) and the presence of the PPRE (dashed box) are indicated. (B) Comparison of the sequence of the proposed PPRE with the consensus. (C) Electrophoretic mobility shift assay of the muscle CPT I PPRE with PPAR-RXR heterodimers. PPAR α, γ and δ and RXRa were translated in vitro, incubated with the proposed CPT I PPRE labelled probe, and analyzed by electrophoretic mobility shift assay. Additions were as indicated on the top of the figure.

the 5′ flanking region of this gene was linked to a promoter-less bacterial chloramphenicol acetyltrasferase (CAT) gene. These plasmids, pMCPT I-CAT, were introduced into cultured CV1 cells by the calcium phosphate method, with or without an expression vector for PPARs, together with a plasmid that contains the β-galactosidase coding region driven by the SV40 promoter as a control of the efficiency of the transfection. Following transfection, cells were incubated in the presence or absence of a

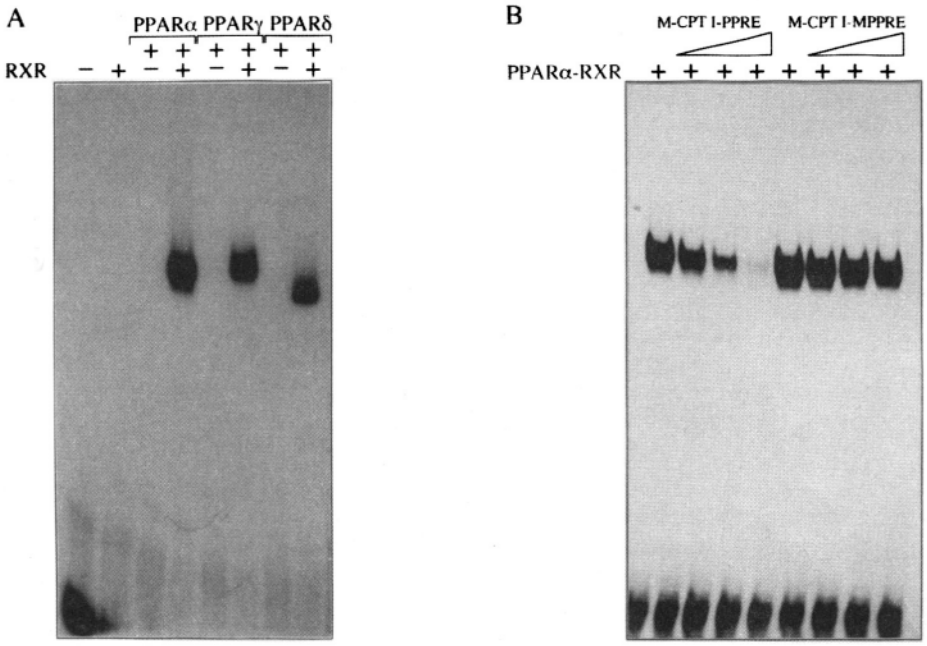

Figure 4. *PPAR-RXR heterodimers bind to human MCPT I promoter.* (A) PPAR α, γ and δ and RXRα were translated in vitro, incubated with the proposed CPT I PPRE labeled probe, and analyzed by electrophoretic mobility shift assay. Additions were as indicated on the top of the figure. Shown in (B) is a competition of the complex PPARα-RXR-PPRE with a 25 to 100-fold molar excess of two different unlabeled oligonucleotides: MCPT I PPRE, containing the proposed PPRE or MCPT I MPPRE with the proposed PPRE mutated.

PPAR activator and after 48 h the cells were harvested and CAT activity was measured.

As can be seen in Table I cotransfection of MCPT I-CAT and PPARs expression vectors led to a marked increase in CAT activity in the presence of the PPAR activators. Surprisingly, even though PPARδ is able to bind the MCPT I PPRE in vitro it does not activate the expression of the chimeric gene even in the presence of linoleic acid as activator.

2.4. Human MCPT I PPRE Confer PPAR Sensitivity in Its Natural Contexts and PPAR Responsiveness to Normally Unresponsive Promoter

The functionality of human MCPT I PPRE was studied in two ways. First, a pair of oligonucleotides containing the human MCPT I PPRE were inserted into pBLCAT2, a plasmid containing the CAT gene under the control of the thymidine kinase gene promoter, generating pTKCAT-MCPT I. As can be seen in Table I this sequence conferred PPAR responsiveness to the otherwise unresponsive thymidine kinase gene promoter. Second, scrambling of the DR-1 (pMCPTI-CAT-M), by site directed mutagenesis obliterates the response to PPAR (see Table I) of human MCPT I promoter. The results demonstrate that this human MCPT I element is able to confer PPARα and γ responsiveness both on its natural context and on a normally unresponsive promoter.

2.5. PPAR Could Mediate Fatty Acid Regulation of Lipid-Metabolism Related Genes in Hepatic and Extra-Hepatic Tissues

Our data indicate that mitochondrial HMG-CoA synthase and muscle CPT I isotype genes are both targets of PPAR. Other genes involved in mitochondrial lipid metabolism, such as acyl-CoA synthetase[11] or medium-chain acyl-CoA dehydrogenase (MCAD),[12] are also target of PPAR. Therefore PPAR is a pivotal factor for lipid home-

Table 1. *PPARα and γ-dependent activation of the human MCPT I.* pMCPT I-CAT (–909 to + 126,[14]), pMCPT I-CAT-M (–909 to +126, containing a 5′-flanking mutated-region by changing nucleotides –769 to –758,[14] or pTKCAT-MCPT I containing the CAT gene under the control of TK gene promoter and a fragment from the 5′ region of the MCPT I containing the proposed PPRE (–782 to –748,[14]), were cotransfected with expression vectors for PPARα, γ and δ into CV1 cells in the presence of LY (30μM), PGJ2 (10μM) or linoleic acid (30μM) respectively as activators of the different isoforms of PPAR. Values of β-galactosidase-normalized CAT activity are expressed relative to the activity in the absence of both PPARs and activators.

	Co-Transfection			
Transfection	none	PPARα + LY	PPARδ + C18:2	PPARγ + PGJ2
MCPT I-CAT	1	4,10	1,17	3,80
MCPT I-CAT-M	1	0,65	0,90	0,86
TKCAT-MCPT I	1	5,1	1,11	4,40

The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; CAT, chloramphenicol acetyltransferase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; NRRE, nuclear receptor responsive element; RXR, retinoid X receptor; hRXRa, human 9-cis-retinoic acid receptor α; mPPARα, mouse peroxisome proliferator-activated receptor a; COUP-TF, chicken ovalbumin upstream-promoter transcription factor; HNF-4, hepatocyte nuclear factor 4; EMSA, electrophoretic mobility shift analysis; tk, thymidine kinase; NEFA, nonesterified fatty acids

ostasis control in which free fatty acids, or their derivatives could activate their own metabolism by regulating gene expression. In this model, a cis-acting element, the PPRE, is present in house-keeping and tissue specific genes, indicating that fatty acid control of gene expression could be active in hepatic and extra-hepatic tissues.

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