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Citrate carrier promoter is target of peroxisome proliferator-activated receptor alpha and gamma in hepatocytes and adipocytes

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ABSTRACT

Citrate carrier (CiC), a mitochondrial inner membrane protein, is an essential component of the shuttle system which transports acetyl-CoA from mitochondria to the cytosol where lipogenesis occurs. CiC is regulated by SREBP-1, a transcription factor that controls the expression of several lipogenic genes. CiC is also implicated in cholesterol synthesis, glycolysis and gluconeogenesis, suggesting that besides SREBP-1 other transcription factors could modulate the expression of its gene. Here, we provide evidences demonstrating that CiC expression is regulated by peroxisome proliferator-activated receptor (PPAR) alpha and gamma in hepatocytes and adipocytes, respectively. CiC expression increased in rat BRL-3A hepatocytes treated with WY-14,643, agonist of PPARα, and in murine 3T3-L1 adipocytes treated with rosiglitazone, agonist of PPARγ. The overexpression of PPARα/RXRα and PPARγ/RXRα heterodimer enhanced CiC promoter activity in BRL-3A and 3T3-L1, respectively. Luciferase reporter gene and gel mobility shift assays indicated that a functional peroxisome proliferator-activated receptor response element (PPRE), identified in the CiC promoter, conferred responsiveness to activation by PPARs. The binding of PPRE of CiC promoter by PPARα and PPARγ *in vivo* was confirmed by ChIP assay in BRL-3A and 3T3-L1 cells, respectively.

1. Introduction

Citrate carrier (CiC) (also known as tricarboxylate carrier), a mitochondrial inner membrane protein, catalyzes electroneutral exchange of a tricarboxylate as citrate for either another tricarboxylate, a dicarboxylate or phosphoenolpyruvate. *Cic* gene, also named *slc25a1*, belongs to the SLC25 gene family coding for mitochondrial carriers. CiC exports acetyl-CoA, mainly deriving from sugar sources, from mitochondria to the cytosol, providing carbon units for fatty acid and cholesterol syntheses (Palmieri, 2004). In this shuttle system, NAD^+ and NADPH, that support glycolysis and lipid biosynthesis respectively, are also supplied (Kaplan and Mayor, 1993). Moreover, CiC may have physiological functions in gluconeogenesis as well (Palmieri, 2004). It has been reported that CiC activity is enhanced in hyperthyroidism (Palmieri, 2004), reduced significantly during starvation (Siculella et al., 2002), hypothyroidism (Siculella et al., 2006) and type-1 diabetes (Gnoni et al., 2010) and can be regulated by insulin (Gnoni et al., 2010). Studies from our laboratory showed that, in parallel with lipogenic enzyme activities, CiC activity and expression is controlled by various nutritional states (Giudetti et al., 2003; Siculella et al., 2002, 2004a,b). While saturated and monounsaturated fatty acid-enriched diet administration to rats was without effect, dietary n-6 and n-3 polyunsaturated fatty acids (PUFA) inhibited *Cic* gene expression at both transcriptional and posttranscriptional level, being n-3 more effective than n-6 PUFA (Siculella et al., 2004a,b). A PUFA response region containing the binding sites for some transcription factors such as sterol regulatory element binding protein-1c (SREBP-1c), Sp1 and NF-Y has been identified in the *Cic* gene promoter (Damiano et al., 2009). SREBP-1c activates the expression of CiC both in hepatocytes (Damiano et al., 2009) and in mammary epithelium (Rudolph et al., 2010).

In liver and in adipose tissue there are other important transcription factors implicated in the lipid homeostasis, such as peroxisomal proliferator-activated receptors (PPARs) (for reviews, see Sugden et al., 2009; Wang, 2010). PPARs are members of the superfamily of nuclear hormone receptors that function as ligand-dependent transcription factors. Upon ligand activation they regulate the expression of genes containing specific response element, called PPRE. Three subtypes of PPARs, termed α, δ (or β) and γ, have been identified (Sugden et al., 2009; Wang, 2010). These

Abbreviations: ChIP, chromatin immunoprecipitation; CiC, citrate carrier; DR, direct repeat; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; PUFA, polyunsaturated fatty acids; RXR, retinoid X receptor.

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receptors heterodimerize with the retinoid X receptor (RXR) and alter the transcription of target genes after binding to PPRE, which consists of a hexameric nucleotide direct repeat of the recognition motif (TGACCT) spaced by one nucleotide (DR-1).

The transcriptional activity of the PPAR subtypes is enhanced by a multitude of ligands, including fatty acids, thiazolidinedione antidiabetic agents, prostaglandins, peroxisome proliferators and fibrate hypolipidemic drugs. PPARγ preferentially binds antidiabetic thiazolidinediones and prostaglandin derivatives, whereas fatty acids more selectively activate PPARδ. Fibrates and to a lesser extent PUFA are activators of PPARα (Sugden et al., 2009; Pyper et al., 2010; Wang, 2010).

PPARα is predominantly expressed in tissues with high catabolic rates of fatty acids and of peroxisomal metabolism such as liver, heart, kidney, intestinal mucosa and brown adipose tissue (Sugden et al., 2009; Pyper et al., 2010; Wang, 2010). It activates genes encoding for enzymes involved in the mitochondrial and peroxisomal fatty acid β-oxidation and microsomal fatty acid α-oxidation pathways, such as cytochrome P450 4A6, medium chain acyl-CoA dehydrogenase, adipocyte fatty acid binding protein aP2, apolipoproteins A-I and A-II, and the acyl-CoA synthetase (Sugden et al., 2009; Wang, 2010; Pyper et al., 2010). PPARα is involved also in cholesterol homeostasis (Chakravarthy et al., 2005), and it activates gluconeogenesis (Patsouris et al., 2004).

PPARγ has two major isoforms, γ1 and γ2, generated from the same gene by alternative splicing (Sugden et al., 2009; Wang, 2010). PPARγ2 is highly expressed in adipose tissue and in steatotic liver of *ob*/*ob* mice, whereas PPARγ1 is present at low levels in many tissues (Tontonoz et al., 1994; Rahimian et al., 2001; Dubuquoy et al., 2002). Often referred to as the "master regulator" of adipogenesis, PPARγ participates in the transcriptional activation of several genes important for adipocyte maturation, lipid accumulation, and insulin-sensitive glucose transport, and coding for aP2, CCAAT/enhancer-binding protein-α (C/EBPα), Perilipin, and GLUT4 (Tontonoz et al., 1994; Wu et al., 1998; Rosen et al., 2002; Arimura et al., 2004).

As CiC works at the intersection of a number of metabolic pathways and processes, here we evaluated whether, besides SREBP-1, other transcription factors might regulate its expression. In this study, we provide the first evidence indicating *Cic* as a target gene of PPARα and PPARγ in hepatocytes and adipocytes, respectively.

2. Materials and methods

2.1. Materials

DMEM (Dulbecco's modified Eagle's medium), calf serum, fetal bovine serum (FBS), penicillin G, Streptomycin, WY-14,643, insulin, dexamethasone, 3-isobutyl-1-methylxanthine are from Sigma. SV Total RNA Isolation System kit, Dual-Luciferase assay kit are from Promega. FuGENE 6 reagent is from Roche Diagnostics. PPARα (sc-9000), PPARγ (sc-7273) and CiC (sc-86392) antibodies are from Santa Cruz Biotechnology.

2.2. Plasmid constructs

The p72Em construct is described in a previous work (Damiano et al., 2009). Two DNA fragments of rat CiC promoter (sizes from −1114 to +35 and −469 to +35 relative to the transcription start site) were obtained by PCR using p72Em as template and the forward primers 1114For 5′-GAATTCGGTACCTGTAGGCTCTCCTCTGCTGC-3′, 469For 5′-GAATTCGGTACCAAGCTCTGTG ATTACAATG-3′, 147For 5′-GAATTCGGTACCTCAGTTTCCCGGCTGGCACG-3′, respectively. The common reverse primer was Co23rev (5′-GCGTCGACCTCGGGTCCGAG-3′). The amplification products were digested with KpnI and SalI, then subcloned into the KpnI and XhoI sites of pGL3 basic vector (Promega). p72Em(PPRE625mut) construct with the mutated PPRE site at −625 bp of the rat CiC promoter was created by site-directed mutagenesis. The mutation was obtained by using the p72Em as template for PCR reactions, the primers PPREmutFor (5′-AGGAGGTACAACGACCAACTTAATA-3′), PPREmutRev (5′-TACCTCCTACCCACACAATCACCAG-3′), and two end primers 1484For and Co23rev (Damiano et al., 2009). All the constructs were sequenced to confirm the accuracy, by using BigDye™ Terminator cycle sequencing kit (Applied Biosystem). pcDNA3-PPARγ2 was kindly provided by Oshima T., Tokusima Bunri University, Kagava, Japan.

2.3. Cell culture

Rat BRL-3A hepatocytes and murine 3T3-L1 preadipocytes were cultured in DMEM supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), and 10% (v/v) FBS or 10% (v/v) calf serum, respectively. Cells were incubated at 37 °C under 5% CO_2 atmosphere. After reaching confluence (designed as day 0), the differentiation of preadipocytes in mature adipocytes was induced by culturing cells in medium containing 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine. After 48 h, the medium was replaced with DMEM supplemented with 10% FBS and 1 μg/ml insulin. The cells were subsequently re-fed every 48 h with DMEM supplemented with 10% FBS and 1 μg/ml insulin.

2.4. Isolation of RNA from and BRL-3A and 3T3-L1 cells and real-time qPCR analysis

Total RNA from BRL-3A and 3T3-L1 cells was isolated using the SV Total RNA Isolation System kit. The reverse transcriptase reaction (20 μl) and quantitative gene expression analysis were performed as in (Damiano et al., 2009). The primers used for real-time PCR analysis were the following: CiCfor (5′-CGAAATCTGCATCACCTTCC-3′); CiCrev (5′-CATGGCTGCGGACAGTTTGC-3′); PPARγ2for (5′-CCAGAGCATGGTGCCTTCGCT-3′); PPARγ2rev (5′-TCAGCAACCATTGGGTCAGC-3′); aP2for (5′-CTTTGTGGGAACCTGGAAGC-3′); aP2rev (5′-TCATCGAATTCCACGCCCAG-3′). 18S rRNA was used for normalization.

2.5. Western analysis

80 μg of proteins from BRL-3A or 3T3-L1 cells was separated by SDS–PAGE. After electrophoretic transfer to nitrocellulose, blot was probed with antibodies directed against CiC and β-actin. The detection system employed was the ECL Plus™ Western Blotting Reagents (GE Healthcare).

2.6. Transient transfection and luciferase reporter assays

The reporter plasmids were transfected into hepatocytes or preadipocytes using the FuGENE 6 reagent (Damiano et al., 2009). For transcriptional activation by pSG5-mPPARα and pSG-mRXRα heterodimer, BRL-3A cells were transiently co-transfected with one of the reporter plasmids described above together with either pSG5-mPPARα and pSG-mRXRα or an empty control vector (pSG5). Analogously, to evaluate the effect of PPARγ/RXRα overexpression on the CiC promoter activity, 3T3-L1 cells were transiently co-transfected with one of the reporter plasmids together with either pcDNA3-mPPARγ2 and pSG-mRXRα or an empty control vector (pSG5). In all the transfections, the reference plasmid pGL4.73 coding for *Renilla* luciferase, was also used as control for transfection