Peroxisomal-proliferator-activated receptor α activates transcription of the rat hepatic malonyl-CoA decarboxylase gene: a key regulation of malonyl-CoA level

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MCD (malonyl-CoA decarboxylase), which catalyses decarboxylation of malonyl-CoA, is known to play an important role in the regulation of malonyl-CoA concentration. Recently, it has been observed that the expression of MCD is significantly decreased in the hearts of the PPARα (peroxisome-proliferator-activated receptor α) ($-/-$) mice, where the rate of fatty-acid oxidation is decreased by the increased malonyl-CoA level [Campbell, Kozak, Wagner, Altarejos, Dyck, Belke, Severson, Kelly and Lopaschuk (2002) J. Biol. Chem. **277**, 4098–4103]. This suggests that MCD may be transcriptionally regulated by PPARα. To investigate whether PPARα is truly responsible for transcriptional regulation of the rat MCD gene, transient reporter assay was performed in CV-1 cells. The promoter activity was increased by 17-fold in CV-1 cells co-transfected with PPARα/retinoid X receptor α expression plasmid. In sequence analysis of the promoter region, three putative PPREs (PPAR response elements) were identified, and promoter deletion analysis showed that PPRE2 and PPRE3 were functional. Electrophoretic mobility-shift assays revealed that PPARα/retinoid X receptor α heterodimer indeed bound to the two PPREs, and the binding specificity of PPARα on PPRE was also confirmed by experiments with mutated oligonucleotides. These results indicate that the elements behaved as a responsive site to PPARα activation. MCD mRNA levels in WY14643-treated rat hepatoma cells as well as in the liver of fenofibrate-fed Otsuka Long-Evans Tokushima fatty rats were also found to be increased, suggesting that PPARα can activate the rat hepatic MCD transcription by binding to the PPREs in the promoter. We propose that MCD performs an important role in understanding the regulatory mechanism between activated PPARα and fatty-acid oxidation by altering the malonyl-CoA concentration.

Key words: malonyl-CoA decarboxylase, peroxisome-proliferator-activated receptor α (PPARα), peroxisome-proliferator-activated receptor α response element (PPRE), promoter, transcription.

INTRODUCTION

PPARα (peroxisome-proliferator-activated receptor α) is a member of the nuclear hormone receptor superfamily and is a fatty acid-activated transcription factor that plays a pivotal role in the transcriptional regulation of genes involved in cellular lipid metabolism [1–3]. PPARα is highly expressed in tissues with high rates of fatty-acid oxidation, such as brown fat, liver and heart [4]. PPARα activated by natural or synthetic fatty acids binds to PPREs (PPAR response elements) as a heterodimer with RXR (retinoid X receptor) in the 5′-flanking region of target genes and regulates the transcription of these genes [5,6].

Recent study with PPARα ($-/-$) mice suggested that PPARα could regulate expression of MCD (malonyl-CoA decarboxylase) [7], which catalyses decarboxylation of malonyl-CoA. This enzyme was first characterized from the goose uropygial gland and was proposed to play a role in the biosynthesis of multi-branched fatty acids in the gland [8]. In the meantime, the enzyme in rat liver mitochondria [9,10] and peroxisome [11] was proposed to be responsible for detoxification of those organelles. Previously, much attention has been given to MCD, since it plays an important role in the regulation of intracellular malonyl-CoA level in skeletal muscles [12]. Malonyl-CoA is well known to be an elongating agent for fatty-acid biosynthesis, but it also acts as a modulator of fatty-acid oxidation by inhibiting CPT-1 (carnitine palmitoyltransferase-1) [13–15], which transfers LCFA-CoA (long-chain fatty acyl-CoA) into the mitochondria where they are oxidized [16]. However, the rate at which the concentration of this potential signal can be changed is yet to be studied. For malonyl-CoA to play a convincing role as a signalling molecule, its cellular concentration has to be susceptible to rapid modulation through changes in the rates of formation and/or removal. The malonyl-CoA concentration changes under different physiological conditions [17]. In skeletal muscles, it is decreased during starvation and increased after 24 h of re-feeding [18]. Malonyl-CoA levels in liver are decreased in times of nutritional deficiency or diabetes, which results in limited synthesis of fatty acids and up-regulation of fatty-acid oxidation [19,20]. MCD may be responsible for the decrease in malonyl-CoA level by degradation of malonyl-CoA into acetyl-CoA. In spite of the importance of MCD in the regulation of fatty-acid oxidation, the mechanism involved in regulating the expression of this enzyme has not been clearly elucidated. However, ACC (acetyl-CoA carboxylase), which catalyses conversion of acetyl-CoA into malonyl-CoA, has been intensively investigated [21–23]. Insulin and glucose appear to activate ACC_{β} in muscles by increasing the cytosolic concentration of citrate, an allosteric activator of ACC_{β} and a precursor of its substrate, cytosolic acetyl-CoA. In

Abbreviations used: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; 9-*cis*-RA, 9-*cis*-retinoic acid; CPT-1, carnitine palmitoyltransferase-1; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LCFA-CoA, long-chain fatty acyl-CoA; MCD, malonyl-CoA decarboxylase; NEFA, non-esterified fatty acid; OLETF, Otsuka Long-Evans Tokushima fatty; PPARα, peroxisome-proliferator-activated receptor α; PPRE, PPAR response element; RT, reverse transcriptase; RXRα, retinoid X receptor α; TG, triacylglycerol.

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contrast, decreases in malonyl-CoA concentration and increases in fatty-acid oxidation in muscle during exercise have been linked to decreases in ACC_β activity, attributable to its phosphorylation and inhibition by the $\alpha 2$ isoform of AMPK (AMP-activated protein kinase) [24]. AMPK is well known as a key enzyme responsible for the phosphorylation of ACC [25,26]. Activation of MCD by phosphorylation with AMPK was also reported [27,28]. In addition to inactivation of ACC and activation of MCD by phophorylation, transcriptional regulation of MCD but not ACC by PPAR was also reported [7]. In the hearts of PPARα $(-/-)$ mice, malonyl-CoA level was found to be increased, thereby contributing to the low rate of palmitate oxidation observed in these hearts. This increase in malonyl-CoA level is neither due to an increase in ACC expression or activity nor due to alterations in AMPK control of ACC activity. However, the increase in malonyl-CoA level can be explained by the decreased expression and activity of MCD in the hearts of mice lacking PPARα, suggesting that MCD is under the transcriptional control of PPARα. In a recent report, we identified the presence of putative PPRE in rat MCD promoter [29]. In the present study, we found that the expression of MCD is truly activated by binding of PPARα/RXRα on two PPRE sites of rat MCD.

MATERIALS AND METHODS

Materials

WY14643 was purchased from Tocris Cookson (Bristol, U.K.). Fenofibrate and 9-*cis*-RA (9-*cis*-retinoic acid) were purchased from Sigma–Aldrich Korea (Seoul, South Korea). WY14643 (10 mmol/l) and 9-*cis*-RA (2 mmol/l) were prepared in 50 % (v/v) ethanol and 50 % (v/v) DMSO respectively. pGL3-basic, Luciferase Assay System and the TNT T7 Quick Coupled Transcription/Translation System were from Promega (Madison, WI, U.S.A.). The anti-PPARα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA, U.S.A.).

Plasmids

Expression plasmids encoding the mouse PPARα (pCMX-mPPARα) and RXRα (pCMX-mRXRα) were gifts from Dr R.M. Evans and Dr D.J. Mangelsdorf (Howard Hughes Medical Institute, CA, U.S.A.). Construction of the rat MCD/Luc reporter genes has been described previously [29]. 5′-Flanking region of rat MCD spanning $-2240/+158$ bp was cloned into pGL3 basic vector and named pMCD/Luc-2240. For the construction of PPRE-deleted reporters, pMCD/Luc△PPRE1,2,3, pMCD/Luc△PPRE1, pMCD/Luc△PPRE2, pMCD/Luc△PPRE3 and pMCD/Luc△PPRE2,3, *Eco*RV sites were introduced into the appropriate region by site-directed mutagenesis and *Eco*RV-digested fragments were excised. PPRE mutant constructs were generated by introduction of 1–3 bp substitutions using the method of Quik Change site-directed mutagenesis (Stratagene, La Jolla, CA, U.S.A.). The integrity and fidelity of all promoter–reporter constructs thus made were verified by DNA sequencing.

Cell culture and transient transfection

CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5 % CO_2 atmosphere. Transient transfections were performed using lipofectamine™ PLUS reagent (Invitrogen) as described previously [29]. The medium was replaced 24 h after transfection with Dulbecco's modified Eagle's medium containing appropriate ligands or their vehicles (1:1 mixture of DMSO and ethanol). After 24 h of culture, cells were lysed and assayed for luciferase and β-galactosidase activity. Luciferase activities were normalized by β-galactosidase activities to adjust transfection efficiency. All transfection results were presented as means ± S.E.M. for at least three independent experiments. H4IIE rat hepatoma cells were grown on a 10 cm culture dish in RPMI 1640 medium supplemented with 10 % (v/v) foetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5 % CO_2 atmosphere at a cell density that would reach approx. 60 % confluence after overnight culture. After 24 h, the cells were exposed to various concentrations of WY14643 or vehicles (1:1 mixture of DMSO and ethanol) for 24 h, or 20 μM WY14643 for the times indicated in the experiments.

In vitro transcription/translation

cDNAs for PPARα and RXRα were transcribed and translated *in vitro* from the plasmids pTNT-mPPARα and pTNT-mRXRα, using the TNT T7 Quick Coupled Transcription/Translation systems (Promega) according to the manufacturer's instructions. The translation products were confirmed by SDS/PAGE.

EMSA (electrophoretic mobility-shift assay)

Oligonucleotides for EMSA were end-labelled with [γ-^{32}P]ATP (Amersham Biosciences, Seoul, South Korea) by T4 polynucleotide kinase (Promega) and annealed with antisense oligonucleotides. The resulting double-stranded oligonucleotides were purified with Microspin G-50 column (Amersham Biosciences). Approx. 0.2 ng (50 000 c.p.m.) of the oligonucleotide probe was incubated for 20 min at room temperature (25 °C) with 2 μl of *in vitro* transcribed/translated proteins. The reaction buffer contained 25 mM Hepes (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10 % (v/v) glycerol and 1 μg of poly(dI/dC). For competition experiments, 10–100-fold molar excess of the unlabelled double-stranded competitor oligonucleotides were added to the reaction mixtures. For the supershift assay, 2 μg of anti-PPARα antibody (Santa Cruz Biotechnology) was added to the reaction. DNA–protein complexes were separated by 5 % non-denaturing PAGE in 0.5 × TBE (44.5 mM Tris/borate/1 mM EDTA, pH 8.0). Double-stranded oligonucleotides comprising the following sequences were used for binding and competition analysis: MCD–PPRE2, 5′-GTGGGAAAATAGGCAAGAGGCTGAGCAAGTGAC-3′; MCD–PPRE3, 5′-CGGGGCTCTCGGAACCTTTGGCTGCACTTGGCCTCC-3′; MCD–PPRE2mt, 5′-GTGGGAAAATAGGCAAGttGCTGAGCAAGTGAC-3′; MCD–PPRE3mt, 5′-CGGGGCTCTCGGAACgaTTGGCTGCACTTGGCCTCC-3′. The PPRE sequence is underlined and mutated bases are shown in lower-case letters. MCD–PPRE2 and MCD–PPRE3 contain the sequences of rat MCD promoter region from -142 to -123 and -44 to -25 respectively.

Animal studies

OLETF (Otsuka Long-Evans Tokushima fatty) rats ($n = 15$, 4 weeks old) were donated by Otuska Pharmaceuticals (Tokushima, Japan) and fed till 12 weeks. At this age, the subjects were divided into two groups, namely control and treatment groups. Rats of the control group ($n = 8$) were fed with standard rat diet, and those of the treatment group ($n = 7$) were fed with standard rat diet containing fenofibrate (200 $mg \cdot kg^{-1} \cdot day^{-1}$) for 2 weeks. At 14 weeks, all rats were subjected to an oral glucose tolerance test and then killed to determine the enzyme activity and for mRNA analysis. All the rats were well-cared for the entire period of the experiments as per the Guidelines

of Animal Experiments recommended by Korean Academy of Medical Sciences.

Plasma lipid assay

The levels of TG (triacylglycerol), total cholesterol and NEFA (non-esterified fatty acid) in plasma were measured by Infinity Triglycerides Reagent, Infinity Cholesterol Reagent and ACS-ACOD enzyme method (NEFA ZYME-S, Aiken, Japan) respectively.

Assay of MCD activity

Frozen livers (500 mg) were powdered in liquid nitrogen, weighed and then homogenized in a glass homogenizer containing 30 vol. of a buffer comprising 0.1 M Tris/HCl (pH 8.0), 2 mM PMSF, 5 μM aprotinin, 5 μM leupeptin and 5 μM pepstatin A, with the addition of 40 mM β-glycerophosphate, 40 mM NaF, 4 mM sodium pyrophosphate and 1 mM Na_3VO_4 to inhibit phosphatase activity [9,30]. The homogenized livers were then centrifuged at 500 ***g*** for 10 min. Powdered $(NH_4)_2SO_4$ was slowly added to the supernatant with stirring until 40 % saturation was achieved. The mixture was stirred for 1 h on ice and centrifuged at 14 000 ***g*** for 10 min. The supernatant from this spin was treated with additional $(NH_4)_2SO_4$ until 55 % saturation was achieved. The mixture was re-centrifuged at 14 000 ***g***. The resultant pellet fraction was dissolved in 0.1 M Tris/HCl (pH 8.0) and stored at 4 °C for further use. MCD activity was assayed by measuring the amount of $^{14}CO_2$ generated from [3-^{14}C]malonyl-CoA. The reaction mixture contains 10 μmol of Tris/HCl buffer (pH 8.0), 0.01 μmol of dithioerythritol, 0.02 μmol of [3-^{14}C]malonyl-CoA (15 000 c.p.m.), and partially purified MCD was incubated for 10 min at 30 °C in a total volume of 0.1 ml. The $^{14}CO_2$ generated was trapped in 2 M KOH and assayed by liquid-scintillation spectrometry using a counting fluid consisting of 30 % ethanol in toluene containing 4 g of Omnifluor/l. The enzyme activity was calculated as $\mu mol \cdot (mg\ of\ protein)^{-1} \cdot min^{-1}$.

RNA analysis

Total RNA from H4IIE cells was isolated using TRIzol® Reagent according to the manufacturer's instruction (Invitrogen). For semi-quantitative RT (reverse transcriptase)–PCR, first-strand cDNA was synthesized from 2 μg of total RNA in a total volume of 50 μl using oligo(dT) primer and Moloney-murine-leukaemia virus RT (Invitrogen). Reverse transcription reaction mixture (1 μl) was amplified with primers specific for rat MCD and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in a total volume of 25 μl. Linearity of the PCR was tested by amplifying a total of 40 ng of RNA in amplification cycles between 20 and 40. According to this amplification profile, samples were amplified for 30 cycles using the following parameters: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Primers used in PCR were as follows: MCD-sense, 5′-TTGTGAGGCTGTGCACCC-TG-3′; MCD-antisense, 5′-CTGCAGCTCCTTG ACCACTC-3′; GAPDH-sense, 5′-GCTGCCTTCTCTTGTGACAAA-3′; and GAPDH-antisense, 5′-CACGCCACAGCTTTCCAGA-3′. Amplification products were visualized after electrophoresis on a 2 % (w/v) agarose gel.

Total RNA from OLETF rat liver was isolated using TRIzol® Reagent according to the manufacturer's instruction (Invitrogen). Real-time RT–PCR (ABI Prism 7700 Sequence Detection System) was performed with the TaqMan EZ RT–PCR Core Reagents (ABI, Applied Biosystems, Foster City, CA, U.S.A.). The nucleotide sequences for probes as well as forward and reverse primers of the rat transcript have been published previously [31]. Levels of various mRNAs were normalized to those of cyclophilin.

Statistical analysis

All data were expressed as means ± S.E.M. Differences between groups were examined for statistical significance using the Student's t test. $P < 0.05$ denoted the presence of a statistically significant difference.

RESULTS

PPREs of the rat MCD promoter

To identify potential PPREs in the rat MCD promoter, a previously described sequence of the rat MCD promoter from −2240 to +158 was analysed using the program MatInspector (Genomatix Software, Munich, Germany). The transcription start site had been previously identified at −158 relative to the translation start site [29]. Sequence analysis revealed the presence of three putative PPREs on MCD promoter, and these sites were named PPRE1, PPRE2 and PPRE3 as described in Figure 1.

Transactivation of rat MCD promoter by PPARα

To test whether PPARα is responsible for the transcriptional regulation of rat MCD gene, and to examine the validity of the particular direct repeat 1 sites found in the rat MCD promoter, we cloned this promoter (sequence spanning from −2240 to +158) and inserted it into the pGL3-basic reporter vector. Transient transfection of this luciferase reporter into CV-1 cells in combination with PPARα and RXRα expression vectors and treatment with the RXRα ligand 9-*cis*-RA (1 μM) and the PPARα ligand WY14643 (20 μM), induced reporter activity (Figure 2A). A maximal 17-fold increase in reporter activity was observed after the addition of both receptor expression vectors and ligands. Furthermore, transfection experiments with increasing concentrations of WY14643 induced the reporter activity in a dose-dependent manner in the presence of RXRα and 9-*cis*-RA (Figure 2B). Results obtained from reporter assay in CV-1 cells suggested that PPARα is an important transcriptional regulator in the MCD promoter.

Identification of a functional PPRE in rat MCD promoter

To demonstrate further the functionality of these PPRE sites, we prepared 5′-serial deletion constructs and monitored the promoter activity in the presence of PPARα and RXRα expression vectors and their ligands in CV-1 cells. The 5′-serial deletion study suggested that the activation required the sequences between −205 and −15 bp of the rat MCD promoter (results not shown). To determine the location of the region responsible for the transactivation by PPARα, we prepared several deleted promoter-luciferase constructs lacking sequences from −233 to −12 (pMCD/Luc△PPRE1,2,3), from −233 to −167 (pMCD/Luc △PPRE1), from −167 to −102 (pMCD/Luc△PPRE2) and from −102 to −12 (pMCD/Luc△PPRE3) (Figure 3A). The construct pMCD/Luc△PPRE1,2,3 lost its responsiveness to PPARα and pMCD/Luc△PPRE1 retained PPARα responsiveness, whereas pMCD/Luc△PPRE2 and pMCD/Luc△PPRE3 slightly lost their responsiveness to PPARα stimulation, but not to the extent of complete abolition. Importantly, the activation of PPRE1-deleted construct was almost identical with that of the wild-type promoter. Thus we could narrow down the location of PPRE to the regions of −167/−102 (PPRE2) and −102/−12 (PPRE3). Then, we generated the deletion construct pMCD/Luc△PPRE2,3

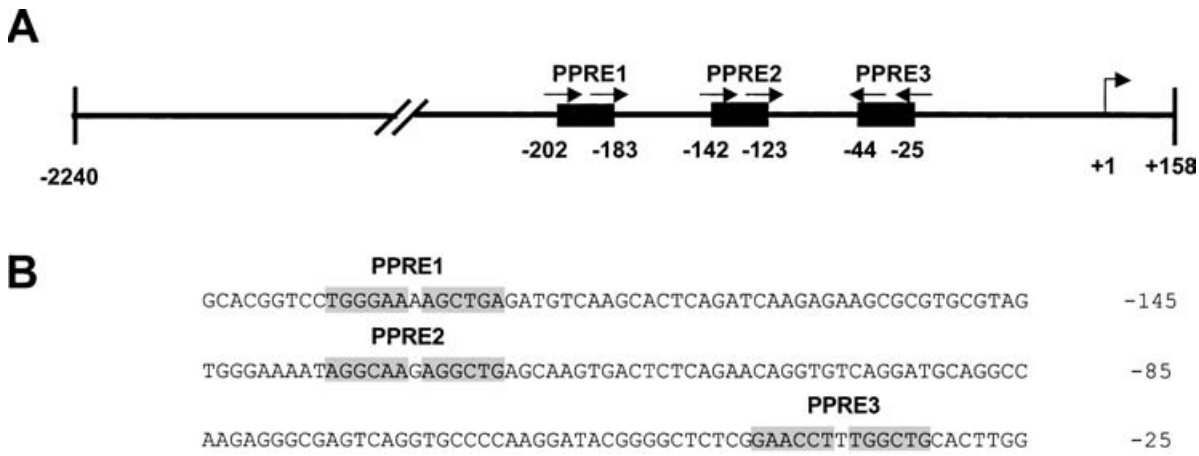

Figure 1 PPREs on rat MCD promoter

(**A**) A schematic representation of the cloned rat MCD promoter (nt -2240 to $+158$). Transcription start site is marked by $+1$. The nucleotide positions of the black box points to the localization of the PPRE. (**B**) Sequence of the nucleotides surrounding the rat MCD PPRE. The two half sites are encircled by boxes.

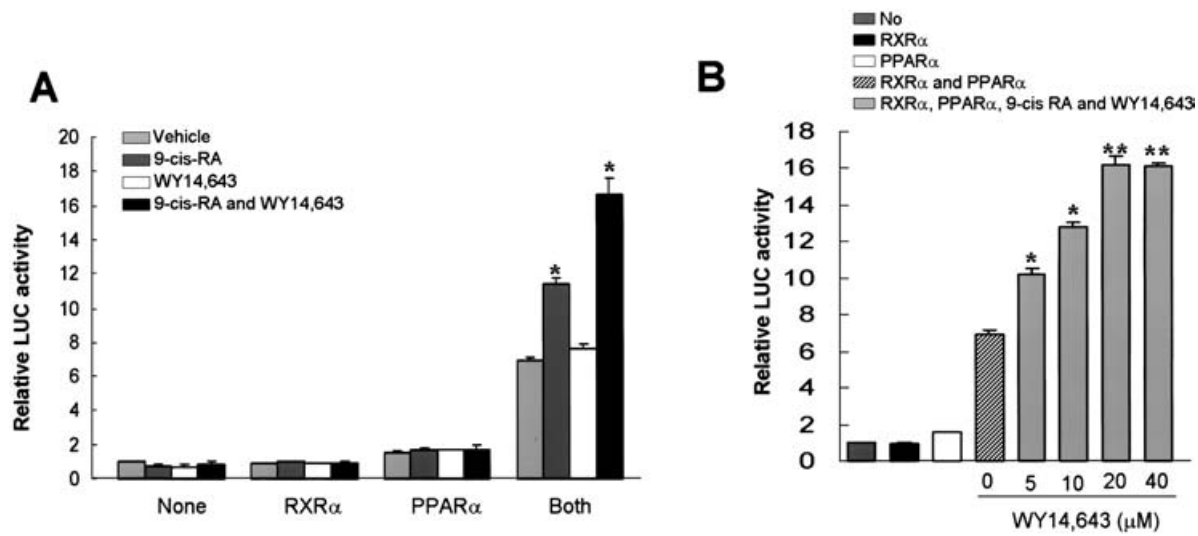

Figure 2 PPARα dependence on rat MCD promoter activity

(**A**) Transient transfection with the full-length rat MCD luciferase reporter into CV-1 cells. The cells were co-transfected with β-galactosidase expression vector (as an internal control) and RXRα and/or PPARα expression vectors as indicated. The medium was supplemented with vehicle (DMSO; light grey), 1 μM 9-*cis*-RA (dark grey), 20 μM WY14643 (white) or both ligands (black). After transfection, the cells were incubated for 48 h. $^{*}P < 0.001$, vehicle-treated versus ligand-treated. (**B**) Dose titration for WY14643 on the rat MCD promoter reporter. For all transfections, the result is representative of at least three individual experiments performed in triplicate. Normalized luciferase activities are shown as means $\pm$ S.E.M. ($n = 3$) and are expressed as fold induction relative to the pMCD/Luc-2240 promoter activity in the pCMX empty vector treated with vehicle. $^{*}P < 0.01$ and $^{**}P < 0.0001$, vehicle-treated versus WY14643-treated.

in which both PPRE2 and PPRE3 were deleted. This construct completely lost its responsiveness to PPARα (Figure 3B).

In addition, the regions from -142 to -123 and from -44 to -25 including PPRE2 and PPRE3 respectively were predicted as a putative PPRE by the TRANSFAC® database (http://www.gene-regulation.com/pub/databases.html#transfac). To confirm that these sites are actually the functional PPRE, we generated mutant constructs pMCD/LucPPRE2mt, pMCD/LucPPRE3mt and pMCD/LucPPRE2,3mt that contained targeted mutation within PPRE (Figure 3A). In the co-transfection experiments, cells transfected with mutant constructs of either pMCD/LucPPRE2mt or pMCD/LucPPRE3mt showed a slight decrease in the activation of MCD promoter in the presence of ligands. However, cells transfected with the double-mutant construct of both PPRE (pMCD/LucPPRE2,3mt) abolished its responsiveness to PPARα (Figure 3B). These results demonstrate that both MCD-PPRE2 and MCD-PPRE3 may be functional in the rat MCD promoter and contribute co-operatively in MCD transcription.

Binding of PPARα/RXRα heterodimer to the MCD PPRE

EMSAs were performed to determine whether the PPARα/RXRα heterodimer indeed binds to the putative PPRE in the rat MCD promoter (see Figure 4). ^{32}P-labelled dimerized oligonucleotides corresponding to the -142 to -123 (PPRE2) and -44 to -25 (PPRE3) were incubated with *in vitro* translated monomeric PPARα or RXRα or with the PPARα/RXRα heterodimer. A unique band was retarded in the presence of both PPARα and RXRα, but not in the presence of either PPARα or RXRα alone. The band was specific for the oligonucleotide, as it disappeared

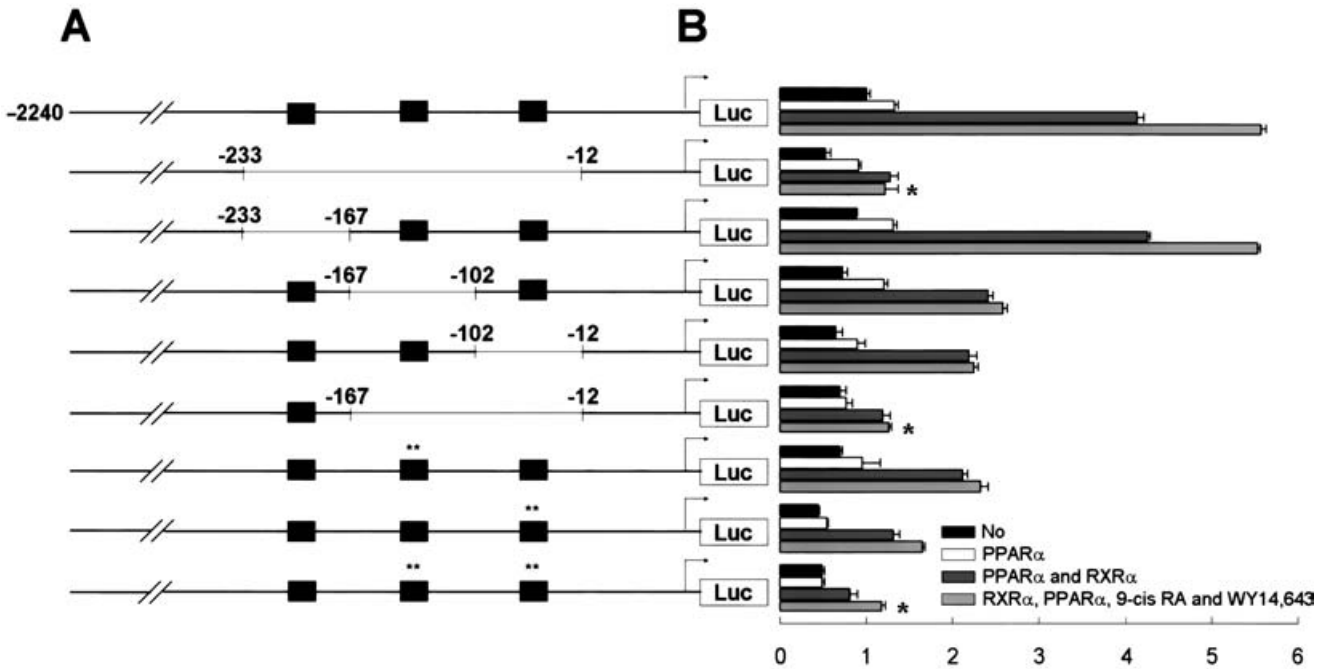

Figure 3 Identification of a PPARα-responsive region in rat MCD promoter

(**A**) A schematic representation of the deletion and the PPRE-mutated constructs of the rat MCD promoter used in transfection experiments. A black box denoted the putative PPREs. The arrow points to the transcription start site. (**B**) Transient transfection of pMCD/Luc-2240, PPRE deletion and PPRE mutation constructs into CV-1 cells. Asterisks (*) indicate the point mutations on PPRE regions. Cells were co-transfected with control plasmid pCMX (black), PPARα expression plasmid (white), PPARα and RXRα expression plasmids (dark grey) and stimulated with 20 μM WY14643 and 1 μM 9-*cis*-RA (light grey). Triplicate transfection was performed in each set of experiments, and relative luciferase activity is presented. Results are given as means ± S.E.M. $*P < 0.0001$, wild-type reporter construct versus mutant type treated with both expression vectors and ligands.

when excess of unlabelled wild-type but not mutated oligonucleotides was added to the mixture. Addition of a PPARα-specific antibody to the mixtures resulted in the disappearance of the retarded band. However, no supershifted band was detected.

Increased expression of the rat MCD in a rat hepatoma cell line, H4IIE, by WY14643

We investigated whether the endogenous MCD expression is induced in response to WY14643 in a rat hepatoma cell line, H4IIE. H4IIE cells were treated with 20 μM WY14643 or vehicle for 24 h. Subsequently, the endogenous MCD mRNA level was quantifed by semiquantative RT–PCR. As shown in Figure 5, the treatment of WY14643 resulted in a 2-fold induction ($P < 0.01$) of the endogenous MCD expression in H4IIE cell lines compared with vehicle-treated control cells. We also tested the effect of WY14643 on the mRNA levels of acyl-CoA oxidase and CPT1, the transcription of which is controlled by PPARα [32,33]. Treatment of H4IIE cells with WY14643 caused a 1.5- and 2.3-fold induction ($P < 0.05$) in acyl-CoA oxidase and CPT1 mRNA levels respectively. These results showed that transcriptional activation of MCD gene by PPARα was physiologically relevant and conferred induction of endogenous MCD mRNA level in rat hepatoma cells.

Fenofibrate increases hepatic mRNA expression and activity of MCD and decreases TG in OLETF rat

To determine whether fenofibrate increases the hepatic mRNA expression and activity of MCD *in vivo*, fenofibrate was administered orally to the OLETF rats at a dose of 200 $mg \cdot kg^{-1} \cdot day^{-1}$ for 2 weeks. OLETF rats showed decreased TG level after the administration. We measured MCD activity and mRNA level in the control and fenofibrate-treated rat livers. The mRNA levels of MCD in liver were significantly increased in the treatment group. Hepatic mRNA expression of MCD increased by 3.2-fold at the end of treatment (Figure 6A). Activity of MCD increased by 2.9-fold on the same day (Figure 6B). This was associated with a decrease in TG level, reaching a 0.55-fold decrease at the same time (Figure 6C). The TG level (50.9 ± 3.8 mg/dl) in the treatment group significantly decreased compared with those (93 ± 14.7 mg/dl) of the control group ($P < 0.03$). The NEFA level (1258.1 ± 106.2 mg/dl) in the treatment group significantly decreased compared with those (1880.4 ± 163.8 mg/dl) of the control group ($P < 0.01$). The total cholesterol level showed no significant difference between the two groups.

DISCUSSION

It has been reported that the low rate of fatty-acid oxidation in the PPARα (−/−) mice hearts is associated with higher concentrations of malonyl-CoA and decreased expression of MCD. In contrast, the expression of ACC, which synthesizes malonyl-CoA, is not altered [7]. These findings suggest that the level of malonyl-CoA may be controlled by the regulation of MCD through PPARα activation.

Previously, we isolated and characterized the promoter of rat MCD gene and identified several putative regulatory-factor binding sites including PPREs [29]. Among these sites, PPRE2 and PPRE3 were responsible for PPARα activation.

The principal finding of this study is that PPARα truly activates transcription of the MCD gene by its direct binding on two PPRE sites in a ligand-dependent manner. This transcriptional regulation of MCD gene expression was also confirmed in rat hepatoma cells as well as in OLETF rat liver.

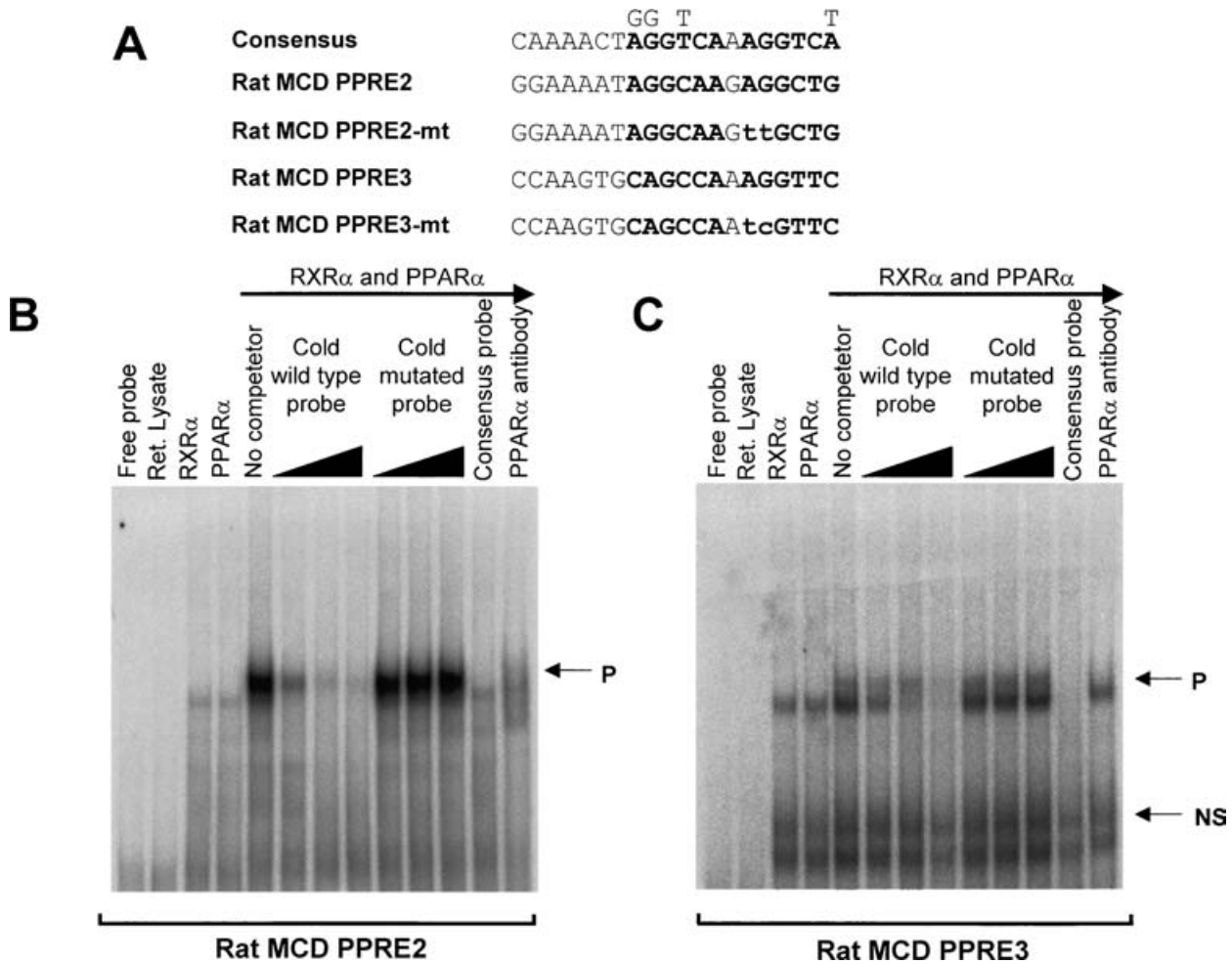

Figure 4 EMSA of the MCD gene PPREs with PPARα/RXRα heterodimers

(**A**) The nucleotide sequence for the different oligonucleotides used in gel-shift studies. Only the upper primer is shown. Each half site in the direct repeat 1 element is indicated in boldface and the base substitution in the mutated oligonucleotides is given in lower-case letters. (**B**) Direct and specific binding of the PPARα/RXRα heterodimer to the PPRE in the rat MCD promoter. The EMSAs were performed with annealed and ^{32}P-radiolabelled rat MCD-PPRE2 oligonucleotides and incubated in the presence of *in vitro* translated PPARα and/or RXRα proteins as indicated. The competition experiment was performed using unlabelled oligonucleotides. For the supershift assay, 2 μg of antibody raised against PPARα was added to the reaction mixture. Consensus oligonucleotides were used for positive control. P, shifted band by PPARα/RXRα heterodimer; NS, non-specific band. (**C**) Direct and specific binding of the PPARα/RXRα heterodimer complex to the rat MCD-PPRE3.

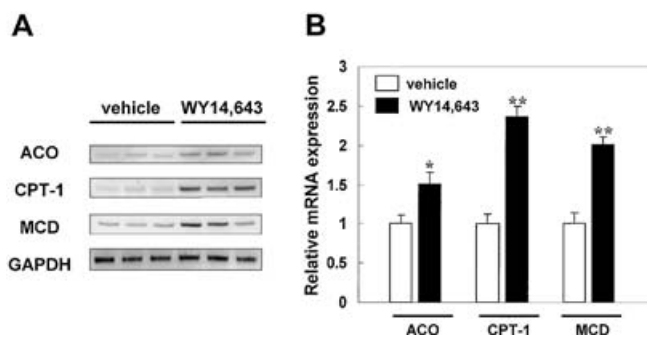

Figure 5 Effects of WY14643 on the expression of MCD mRNA in H4IIE cells

(**A**) Induction of mRNA level in H4IIE cells. Cells were incubated for 24 h with vehicle or 20 μM WY14643. Total RNA was extracted and subjected to mRNA analysis as described in the Materials and methods section. (**B**) Quantification of the GAPDH-normalized mRNA level was shown. The results were expressed as fold increase compared with control treatment with vehicle alone and data were expressed as means ± S.E.M. for three experiments. $*P < 0.05$ and $**P < 0.01$, vehicle-treated versus WY14643-treated.

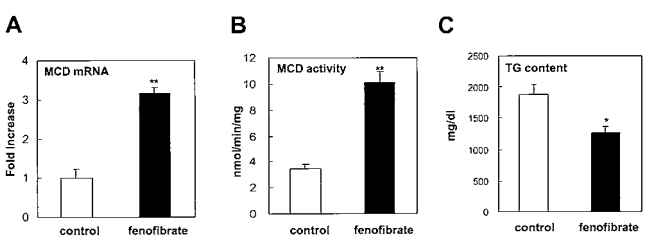

Figure 6 Hepatic MCD mRNA, MCD enzymic activity and TG levels in fenofibrate-fed OLETF rats

OLETF rats were orally administered either vehicle (white bar) or 200 mg · kg^{-1} · day^{-1} of fenofibrate (black bar) for 2 weeks. (**A**) Total RNAs were isolated from the livers. MCD mRNA expression levels were determined by real-time quantitative RT–PCR and normalized to cyclophilin. Data are represented as mRNA expression relative to the vehicle control. (**B**) Rats were killed and livers were homogenized in 0.1 M Tris/HCl (pH 8.0) buffer. $(NH_4)_2SO_4$-purified enzyme was assayed for MCD activity. (**C**) Plasma TG level was analysed. Results are presented as means ± S.E.M. for 8 (control) or 7 (treated) values. $*P < 0.01$ and $**P < 0.0001$, control versus fenofibrate-treated.

Malonyl-CoA is an inhibitor of CPT1, the key regulatory enzyme involved in the first committed step of fatty-acid oxidation. Malonyl-CoA levels are precisely regulated in various nutritional states [19]. Inhibition of CPT1 by malonyl-CoA leads to a decrease in the uptake of fatty acids into the mitochondria, which results in decreasing mitochondrial fatty-acid oxidation [13–15]. Recent studies on the regulation of malonyl-CoA level have focused in two ways: synthetic and degradative. ACC is generally considered to be the rate-limiting enzyme in the synthesis and regulation of malonyl-CoA. On the other hand, it has been strongly suggested that an increase in MCD activity can be the factor contributing to a decrease in malonyl-CoA and increase in fatty-acid oxidation in muscle tissue [34]. Moreover, the activity of MCD is highest in tissues such as heart and liver, which are known as the major organs of fatty-acid oxidation [35]. Also, in pathophysiological states such as diabetes or hyperlipidaemia, where lipid metabolism is altered, MCD has an important role in the regulation of fatty-acid metabolism [36]. These reports support the concept that MCD is involved in regulating the rate of fatty-acid oxidation within the cell by altering the cytoplasmic malonyl-CoA level. So far, it was reported that phosphorylation of MCD by AMPK activator increases the enzyme activity, which is linked to post-translational regulation of MCD in skeletal and cardiac muscles. However, little is known about the molecular mechanism of MCD expression and transcriptional regulation of MCD gene in mammalian cells, except the gene in goose. In goose uropygial gland, a large amount of MCD is localized in the cytosol and a small amount in mitochondria. It has been reported that there are two promoter regions, one for the transcription of cytosolic protein and the other for mitochondrial, although how they are alternatively regulated has not been clearly elucidated yet [37]. In previous work, however, we identified a single transcription-initiation site and not multiple initiation sites in rat [29]. In the present study, we provide the evidence that MCD is transcriptionally regulated by PPARα in OLETF rats. OLETF rats display hypertriglyceridaemia at an early age, develop insulin resistance and subsequently glucose intolerance in adulthood [38]. Thus it is a useful animal model for analysing the molecular mechanism of fatty-acid metabolism and Type II diabetes with obesity. In these rats, malonyl-CoA level in liver is increased [39]. When the concentration of malonyl-CoA is increased, LCFA-CoA transport into the mitochondria decreases and more LCFA-CoA is available in the cytosol for TG synthesis. In the liver of hyperlipidaemic rats, fenofibrate, which is a hypolipidaemic drug that activates PPARα, increases mitochondrial β-oxidation and decreases the plasma level of TG and NEFA [40–42]. We hypothesized that if MCD is directly regulated by PPARα, in fenofibrate-fed OLETF rats, a significant induction of MCD expression may be observed in liver, and subsequently decreased malonyl-CoA level induces mitochondrial β-oxidation. In the treated animal, the enzymic activity and the transcriptional level of MCD were increased, whereas the levels of TG and NEFA were diminished severalfold, as reported previously. Several studies have indicated that fenofibrate increases hepatic β-oxidation, resulting in decreased fatty acids available for TG synthesis [43–45]. Although it is not yet understood which mechanism was involved in the alteration of lipid level, increase in MCD expression reveals that MCD may play a role in lipid catabolism by PPARα.

On the basis of our results, PPARα agonists activate MCD expression *in vitro* in hepatoma cells and *in vivo* in OLETF rat. Although further functional studies are needed, our findings have important implications in understanding the physiological phenomena concerning the cellular malonyl-CoA level and, for the first time, demonstrate the molecular mechanism of transcriptional regulation of MCD gene by PPARα.

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