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Identification of a functional peroxisome proliferator-activated receptor (PPAR) response element (PPRE) in the human apolipoprotein A-IV gene

Michiaki Nagasawa *, Tomoko Hara, Ai Kashino, Yunike Akasaka, Tomohiro Ide, Koji Murakami

Discovery Research Laboratories, Kyorin Pharmaceutical Co., Ltd., 2399-1 Nogi-machi, Shimotsuga-gun, Tochigi 329-0114, Japan

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ABSTRACT

Peroxisome proliferator-activated receptor-α (PPARα) is a key regulator in hepatic lipid metabolism and is a potential therapeutic target for dyslipidaemia. We reported previously that human hepatic apoA-IV is a highly sensitive gene up-regulated by the PPARα agonist KRP-101 (KRP), suggesting that induction of apoA-IV expression is one of the mechanisms underlying the decrease in triglycerides and elevation of HDL observed with PPARα agonist treatment. However, the mechanism of transcriptional regulation of apoA-IV by PPARα activation remains unclear. To clarify whether the apoA-IV promoter is regulated directly by PPARα, we analysed the apoA-IV promoter region by transient transfection assay in the human hepatocellular carcinoma cell line, HepG2. Co-transfection assay of unilateral deletions of apoA-IV promoter construct with human PPARα/RXRα showed that the region from −3279 to −2261 of the apoA-IV promoter includes key sites for transactivation by PPARα/RXRα. Sequence analysis suggested three putative PPAR response elements (PPREs) in this region. Electrophoretic mobility shift assay (EMSA) showed that a PPRE located from −2979 to −2967 can bind to PPARα/RXRα. Moreover, site-directed mutagenesis experiments indicated that the −2979/−2967 PPRE plays an essential role in transcriptional regulation of apoA-IV by PPARα. Chromatin immunoprecipitation (ChIP) assay confirmed that ligand-induced binding of PPARα to endogenous −2979/−2967 PPRE. These results indicate that human apoA-IV is regulated directly by PPARα *via* the −2979/−2967 PPRE.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily [1] and act as important transcriptional regulators involved in lipid and glucose homeostasis [2]. There are three PPAR subtypes: PPARα (NR1C1), PPARγ (NR1C3) and PPARδ (NR1C2) [3]. PPARα is expressed predominantly in tissues that have a high lipid catabolic activity, such as the liver, heart, kidney and muscle [4–6], and regulates the transcription of numerous genes encoding proteins involved in lipid metabolism [7]. After activation, PPARα forms a heterodimer with the retinoid X receptor, RXR (NR2B1), and binds to PPAR response elements (PPREs) that are typically organised as direct repeats of the core motif PuGGTCA within the 5′-flanking regions of target genes, and leads to modulation of their transcriptional activity [8–10].

Recently, we showed that PPARα agonism up-regulates apoA-IV expression with a high degree of sensitivity in human hepatoma cells, and increases serum apoA-IV level in dogs [11]. ApoA-IV is a 46-kDa glycoprotein synthesised in the intestine and liver, which is present in plasma, interstitial fluid and lymph [12–15], and circulates freely or is associated with chylomicrons and HDLs [12]. Previous reports suggested that apoA-IV is correlated with control of plasma HDL levels [16–18], lipoprotein lipase activity [19], appetite in response to fat in the diet [20,21] and protection against atherosclerosis [16,22]. Therefore, up-regulation of apoA-IV induced by PPARα agonism may play a key role in the pharmacological effects of PPARα agonists, such as decreasing triglyceride levels and elevation of HDL, and is one of the beneficial effects of therapy for atherosclerosis as well as dyslipidaemia.

However, the mechanism of transcriptional regulation of human apoA-IV by PPARα activation has not been investigated. In this study, to identify the PPARα-dependent regulatory region in the human apoA-IV promoter, we analyzed the promoter region of human apoA-IV by transient transfection experiments in the human hepatocellular carcinoma cell line, HepG2, and by electrophoretic mobility shift assays (EMSAs) *in vitro*. Then, agonist-responsiveness of identified PPRE was confirmed by transient transfection and chromatin immunoprecipitation (ChIP)

Abbreviations: apo, apolipoprotein; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; HDL, high density lipoprotein; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element.

* Corresponding author. Tel.: +81 280 56 2201; fax: +81 280 57 1293.
E-mail address: michiaki.nagasawa@mb.kyorin-pharm.co.jp (M. Nagasawa).

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experiments, using KRP-101 (KRP), a highly potent and selective human PPARα agonist, and up-regulates expression of apoA-IV in human hepatoma cells [11].

Here, we report a functional PPRE located in the human apoA-IV promoter, which is a critical region for transactivation of the apoA-IV gene by PPARα.

2. Materials and methods

2.1. Chemicals

The PPARα agonist, KRP-101 (KRP), was synthesised by Kyorin Pharmaceutical Co., Ltd. (Tochigi, Japan). Fenofibric acid was obtained from Tyger Scientific (Ewing, NJ, USA).

2.2. Plasmids

Human apoA-IV promoter fragments (−4282/+9) were amplified by PCR from human genomic DNA (Clontech, Mountain View, CA, USA) with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) using the forward primer 5′-AAACGCGTAGGTCAGGACATGTGGGTAGAG-3′ and reverse primer 5′-GCCAGATCTGTGGGAACTGACTGAAGCTCAGAGCCAGCCAGA-3′. The PCR product was digested with MluI and BglII and cloned into the reporter plasmid pGL3-Basic (Promega, Madison, WI, USA), to generate −4282/+9 hAIV-LUC. Plasmids were verified by DNA sequencing, and the nucleotide sequences of the human apoA-IV promoter fragment (−4282/+9) have been submitted to GenBank (accession number: EU429935). The plasmid −4282/+9 hAIV-LUC digested with KpnI/NsiI, KpnI/AflII or NruI/NsiI was blunt-ended and ligated to generate −2260/+9 hAIV-LUC, −1380/+9 hAIV-LUC and −4282Δ/+9 hAIV-LUC, respectively. Site-directed mutagenesis of the construct −4282/+9 hAIV-LUC was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA) according to the manufacturer's instructions, with the mutagenic forward primer 5′-ACTGAGTACTGACCATTGCTCCCGCTGCAAGC-3′ and reverse primer 5′-TTGCAGCGGGAGCAATGGTCAGTACTCAGTGC-3′, to generate −4282Mut/+9 hAIV-LUC. A full-length human PPARα expression vector was prepared as reported previously [23]. The full-length human RXRα was isolated from HepG2 cells (ATCC, Manassas, VA, USA) by RT-PCR using the forward primer 5′-GGAATTCATGGACACCAAACATTTCCTGCCGCTC-3′ and reverse primer 5′-CCCAAGCTTCTAAGTCATTTGGTGCGGCGCCTC-3′. The amplified DNA fragment was digested with EcoRI and HindIII, and cloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, USA) to construct a full-length of human RXRα expression vector.

2.3. Cell transfection and reporter assays

For transfection experiments, human hepatocellular carcinoma cells (HepG2) were seeded on 24-well plates at a density of 5×10^4 cells/well and maintained in Dulbecco's modified Eagle's medium supplemented with 10% delipidated foetal calf serum with 5% CO_2. Cells were transfected with 100 ng of firefly luciferase reporter plasmid as indicated above or 3xACO PPRE-LUC including three copies of rat acyl-CoA oxidase PPRE [23,24], 20 ng of *Renilla* luciferase plasmid pRL-TK (Promega, Madison, WI, USA), and the indicated expression vector using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) and Plus Reagent (Invitrogen, Carlsbad, CA, USA) according to the respective manufacturer's instructions. After 2 h, cells were treated with or without the indicated compounds for 48 h, and cell extracts were measured using Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA). Firefly luciferase activity values were divided by *Renilla* luciferase activity values to obtain normalised luciferase activities. The EC_{50} values of the compounds tested were derived by curve-fitting using the Prism program (GraphPad Software, La Jolla, CA, USA).

2.4. Electrophoretic mobility shift assay (EMSA)

Human PPARα and RXRα proteins were generated from the full-length human PPARα and RXRα expression vectors, respectively, using a coupled *in vitro* transcription/translation system (Promega, Madison, WI, USA). Double-stranded oligonucleotides used in EMSA were labelled with [α-^{32}P]dCTP using Klenow fragment, followed by purification on Sephadex G25 columns. The labelled probes were incubated with 4 μl of *in vitro*-translated protein in 15-μl mixtures containing 10 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol and 26.6 μg/ml poly(dI-dC) for 30 min at room temperature. The DNA–protein complexes were resolved by 4.5% PAGE at 90 V for 1.5 h at room temperature. Gels were dried and analyzed using a Typhoon8600 (Molecular Dynamics, Fairfield, CT, USA).

2.5. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using a Chromatin Immunoprecipitation Kit (Lake Placid Biologicals, Lake Placid, NY, USA). HepG2 cells were seeded onto 100-mm dishes and maintained in Dulbecco's modified Eagle's medium supplemented with 10% delipidated foetal calf serum with 5% CO_2. Cells were transfected with 300 ng of the full-length human PPARα expression vector using Lipofectamine LTX and Plus Reagent according to the manufacturer's instructions. After transfection for 20 h, cells were treated with 0.1% DMSO or 10^{-8} M KRP for 30 min, then fixed in fresh medium containing 1% formaldehyde for 10 min. Chromatin samples (average length 200–1000 bp) were prepared and processed according to the manufacturer's instructions. Immunoprecipitation was performed using an antibody directed against PPARα (H-98; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or with rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as a negative control. After immunoprecipitation, associated DNA was amplified with a primer pair covering positions −2979 to −2967 (PPRE2) (forward 5′-TGCTCTCGGACATGCTCAAATGG-3′ and reverse 5′-GTCCCAACCCTGCTCCTGAATC-3′). PCR products were visualised on 8% polyacrylamide gels stained with ethidium bromide.

3. Results

3.1. Determination of transcriptional regulatory region by PPARα in the human apoA-IV promoter

The transcriptional activity of PPAR-regulated genes containing PPRE in its promoter region is activated by both PPARα/RXRα overexpression [25]. Therefore, to determine the region in the human apoA-IV promoter responsible for up-regulation by PPARα, unilateral 5′-deletions of the human apoA-IV promoter were linked to the luciferase reporter gene, and analysed in transient transfection assays under conditions of exogenously expressed PPARα, RXRα or both PPARα/RXRα in HepG2 cells. As shown in Fig. 1, the luciferase activities of cells transfected with −4282/+9 hAIV-LUC, but not −2260/+9 hAIV-LUC, −1380/+9 hAIV-LUC or pGL3-Basic were increased when co-transfected with both PPARα/RXRα. In contrast, the intact −4282/+9 apoA-IV promoter lacking the sequence between −3279 and −2261 (−4282Δ/+9 hAIV-LUC) was unresponsive to co-transfection with both PPARα/RXRα (Fig. 1). These results suggest that the transcriptional regulatory region by PPARα is localised in the human apoA-IV promoter between positions −3279 and −2261.

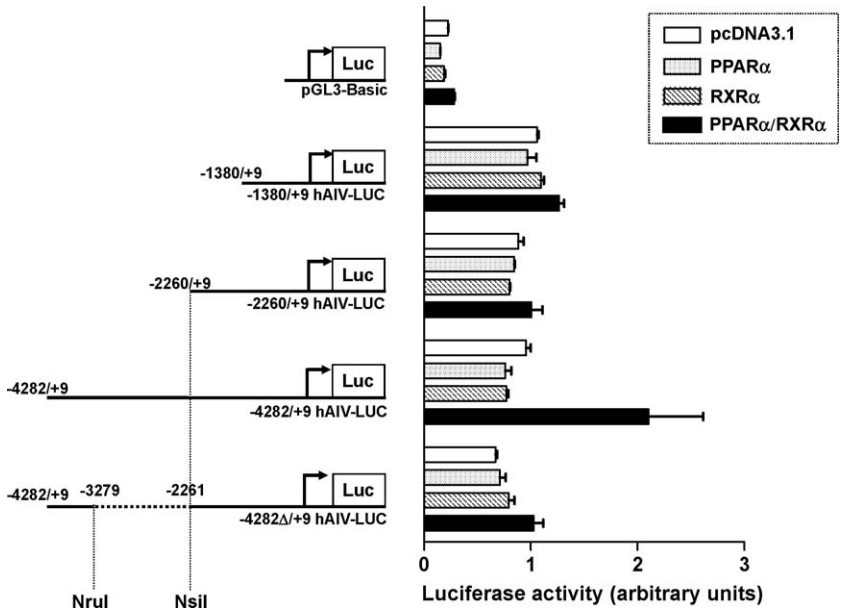

Fig. 1. Determination of a PPARα-responsive region in the human apoA-IV promoter by deletion analysis. HepG2 cells were co-transfected with 100 ng of reporter plasmids containing the firefly luciferase gene driven by progressively 5′-shortened or deleted fragments of the apoA-IV promoter as indicated together with 100 ng of the empty vector pcDNA3.1, a full-length human PPARα expression vector and/or full-length RXRα expression vector. *Renilla* luciferase plasmid pRL-TK (20 ng; Promega) was included in all transfections as an internal control for transfection efficiency. After transfection for 48 h, cell extracts were examined using a Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity values were divided by those of *Renilla* luciferase activity to obtain normalised luciferase activities. Data are presented as the means ± S.E. of three independent experiments.

3.2. Identification of PPRE in human apoA-IV promoter

Next, to identify the PPARα-binding sites in −3279/−2261 human apoA-IV promoter, we performed computer-assisted analysis using TFBIND (http://tfbind.ims.u-tokyo.ac.jp/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). Three putative PPAR-binding motifs were detected from positions −3018 to −3006 (PPRE1), −2979 to −2967 (PPRE2) and −2591 to −2579 (PPRE3), respectively (Fig. 2). Table 1 shows the ranking of the potential sites according to work by Juge-Aubry et al. which demonstrated the

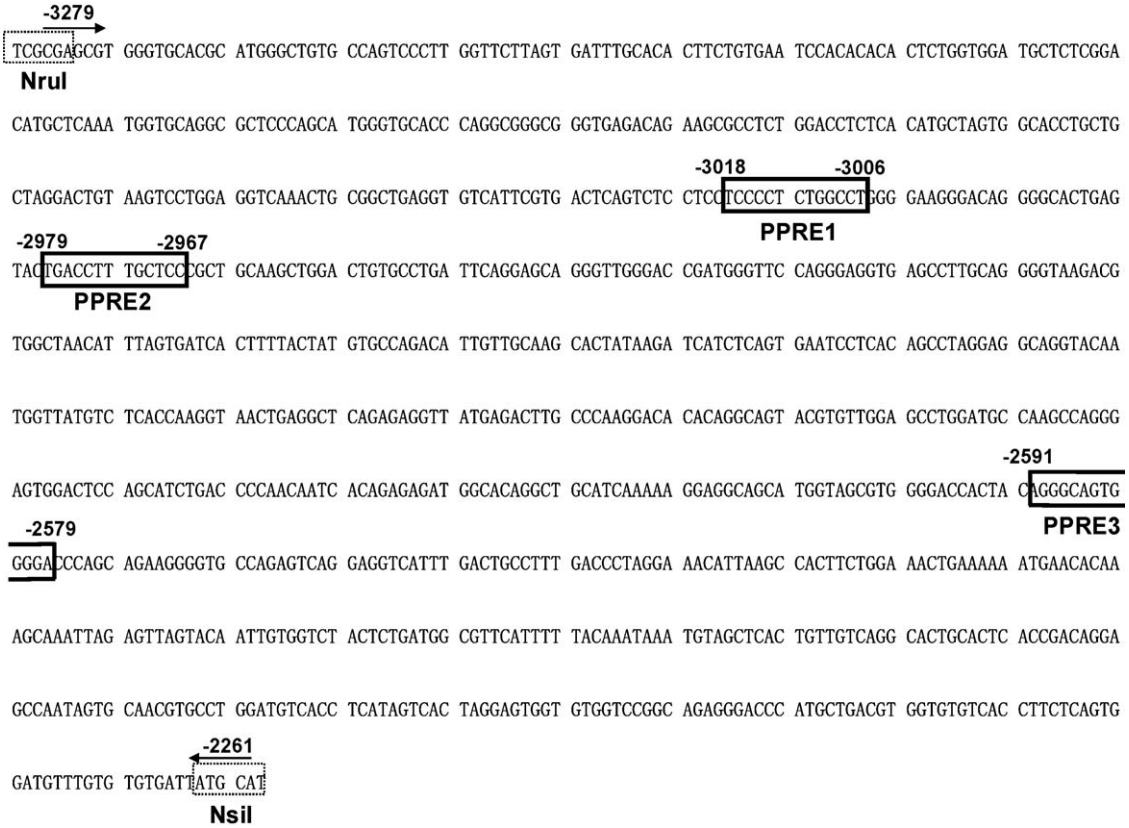

Fig. 2. Nucleotide sequence corresponding to the −3279/−2261 human apoA-IV gene. Three putative PPRE sites, PPRE1, PPRE2 and PPRE3, are boxed.

Table 1
Sequence alignment of the putative and established PPREs [27–30] compared to consensus.

Gene	Localization of PPRE	5'-flank	DR1			Number of matches	Ref.
Consensus		caaaact gg t	AGGTCA	A T	AGGTCA		
ACO	-570/-558	ggggacc	AGGACA	A	AGGTCA	4/7 + 12/13	[27]
apoA-V	-272/-260	agtggga	AGGTTA	A	AGGTCA	1/7 + 12/13	[28]
apoA-I	-212/-197	gggctgc	AGGGCA	G	GGGTCA	3/7 + 10/13	[29]
apoA-II	-734/-716	ttctacc	AGGGTA	A	AGGTTG	2/7 + 9/13	[30]
PPRE1	-3018/-3006	cttcccc	AGGCCA	G	AGGGGA	2/7 + 9/13	
PPRE2	-2979/-2967	tgcagcg	GGAGCA	A	AGGTCA	3/7 + 10/13	
PPRE3	-2591/-2579	ccactac	AGGGCA	G	TGGGGA	3/7 + 8/13	

importance of the 5′-flank region of the PPRE [26]. The PPRE2 ranked highest among three putative PPAR-binding motifs, with 3 out of 7 matches in the 5′-region and 10 out of 13 in the core DR1 compare to the consensus. To clarify which putative PPAR-binding motifs are capable of binding to PPARα, EMSAs were performed using *in vitro*-translated human PPARα, RXRα or both PPARα/RXRα proteins together with radiolabelled oligonucleotides corresponding to PPRE1, PPRE2 and PPRE3 (Fig. 3A). As shown in Fig. 3B, labelled PPRE2, but not PPRE1 or PPRE3, formed a DNA–protein complex in the presence of PPARα/RXRα proteins. An excess of anti-human PPARα antibody abrogated DNA–protein complex formation (Fig. 3B). Formation of the DNA–protein complex was efficiently blocked in a competitive manner in the presence of an excess of unlabelled oligonucleotide corresponding to PPRE2 (Fig. 4). Mutated or deleted PPRE2, PPRE2Mut or PPRE2Del, respectively, did not interfere with DNA–protein complex formation (Fig. 4). These results indicated that PPARα/RXRα heterodimer binds specifically to PPRE2, −2979/−2967 human apoA-IV promoter.

3.3. Requirement of the PPRE2 (−2979/−2967) for transactivation of apoA-IV promoter by PPARα/RXRα overexpression

To clarify whether PPRE2 is a major site for transcriptional up-regulation of human apoA-IV by PPARα, we constructed a human apoA-IV promoter linked to the luciferase reporter gene, in which the PPRE2 sequence was mutated (−4282Mut/+9 hAIV-LUC), and analysed transcriptional activity by transfection assay in HepG2 cells (Fig. 5). The luciferase activity in intact human apoA-IV promoter (−4282/+9 hAIV-LUC) was increased by PPARα/RXRα (Fig. 5). On the other hand, luciferase activity in cells transfected with −4282Mut/+9 hAIV-LUC was significantly decreased (Fig. 5). Again, as shown in Fig. 1, deletion of −3279/−2261 apoA-IV promoter excluding PPRE2 (−4282Δ/+9 hAIV-LUC) also showed almost no increase in luciferase activity by PPARα/RXRα (Fig. 5). These results indicate that PPRE2, −2979/−2967 is a critical region for PPARα/RXRα binding and transactivation of the apoA-IV promoter.

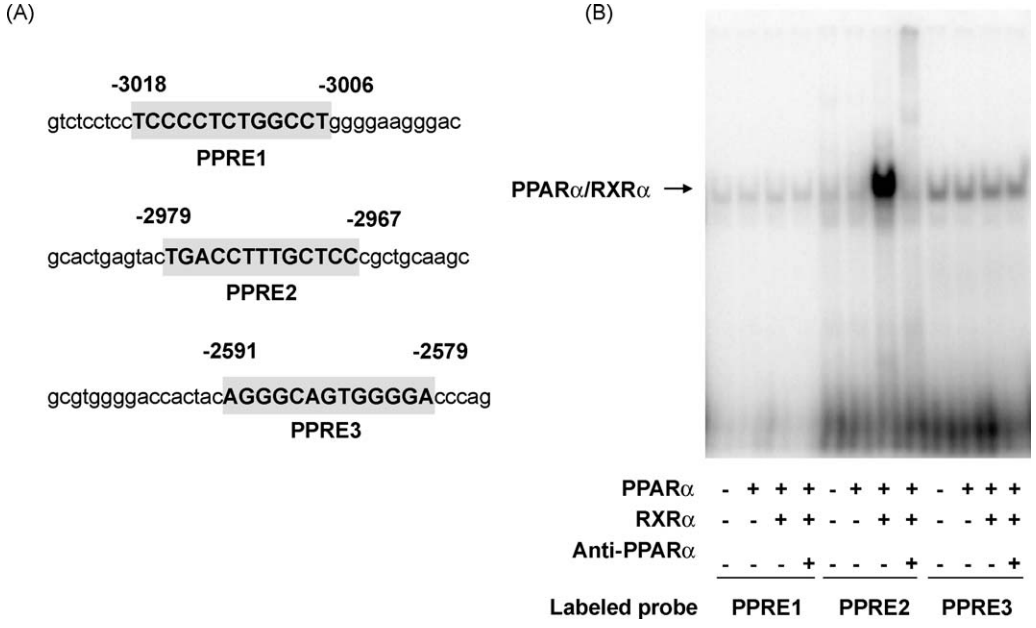

Fig. 3. Characterisation of three putative PPREs in the human apoA-IV promoter. (A) Probes for three putative PPREs, PPRE1, PPRE2 and PPRE3. The grey box indicates the putative PPRE. (B) EMSA. Aliquots of 2 μl of *in vitro*-translated PPARα and/or 2 μl of RXRα were incubated with ^{32}P-labelled PPRE1, PPRE2 or PPRE3 in the presence or absence of 2 μg of anti-PPARα antibody (H-98, sc-9000X; Santa Cruz Biotechnology Inc.). The DNA–protein complexes were resolved by 4.5% PAGE. These experiments were performed three times and the results of one representative experiment are shown.

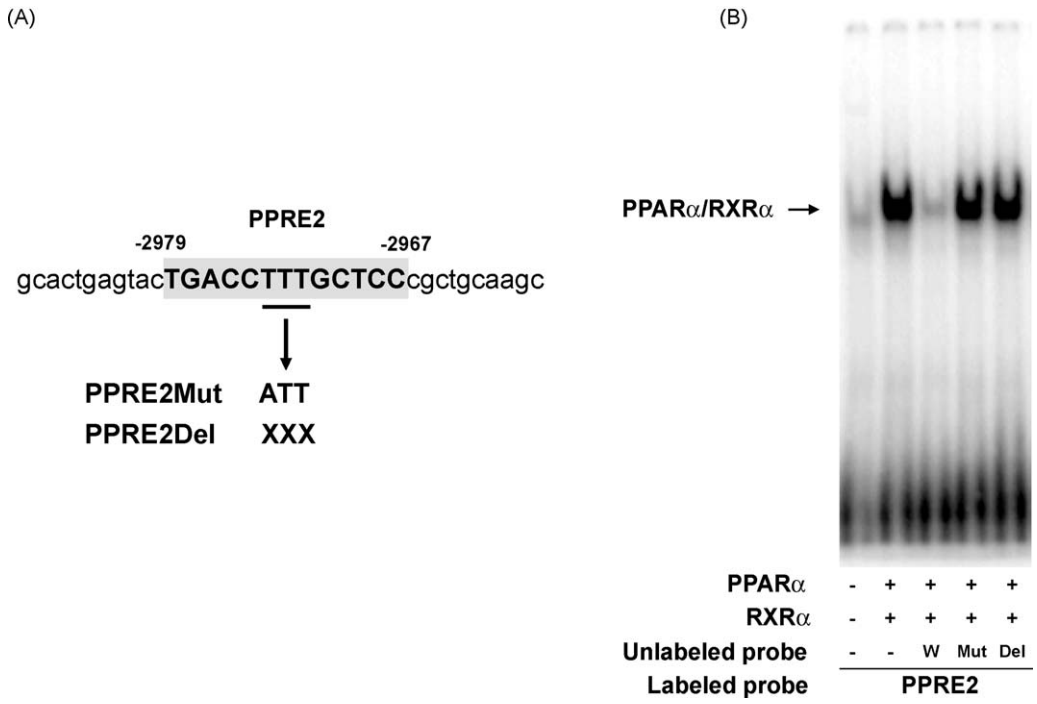

Fig. 4. Functional effects of mutations within the PPRE2 on EMSA. (A) Probes of PPRE2 and its mutants. The putative PPRE was mutated or deleted from 5′-gcactgagtacTGACCTTTGCTCCcgctgcaagc-3′ to 5′-gcactgagtacTGACCATTGCTCCcgctgcaagc-3′ or 5′-gcactgagtacTGACCGCTCCcgctgcaagc-3′, respectively. The grey box indicates the putative PPRE. (B) EMSA. Aliquots of 2 μl of *in vitro*-translated PPARα and 2 μl of RXRα were incubated with ^{32}P-labelled PPRE2 in the presence or absence of a 100-fold molar excess of competitor DNA, intact PPRE2 (W), PPRE2Mut (Mut) or PPREDel (Del). The DNA–protein complexes were resolved by 4.5% PAGE. These experiments were performed three times and the results of one representative experiment are shown.

3.4. PPARα agonist-induced transactivation of apoA-IV promoter via PPRE2 (−2979/−2967)

Next, we examined the essential role of PPRE2 in PPARα agonist-induced transactivation. Transient transfection with a PPARα-responsive reporter gene and PPARα expression vector is a suitable assay to evaluate PPARα agonist-induced transactivation [31]. Therefore, we compared the ligand-induced transactivation of −4282/+9 hAIV-LUC with −4282Mut/+9 hAIV-LUC or −4282Δ/+9 hAIV-LUC by transient transfection assay in the presence of

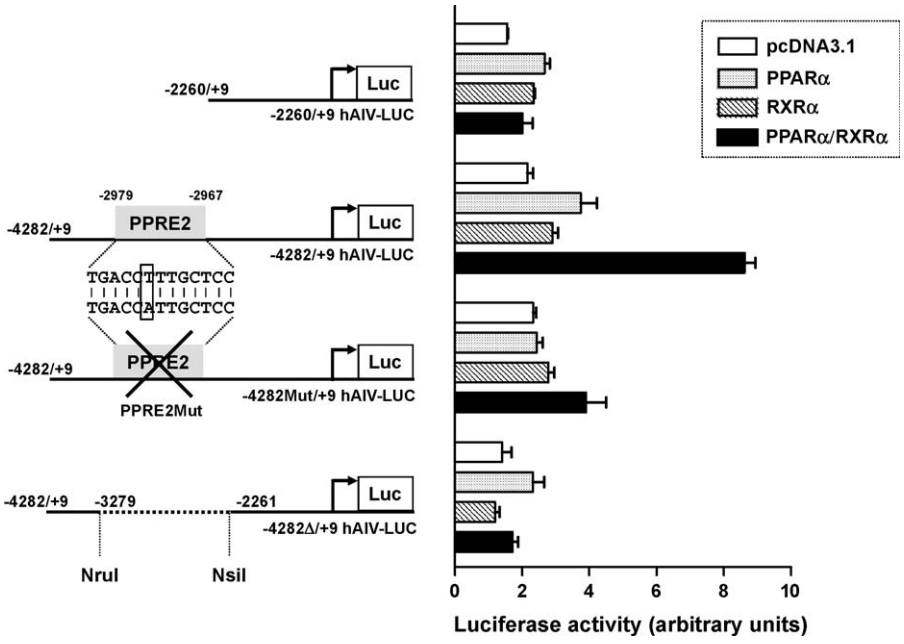

Fig. 5. Functional effects of mutations within the PPRE2 on transcriptional activation of the human apoA-IV promoter by PPARα/RXRα. HepG2 cells were co-transfected with 100 ng of the full-length human PPARα expression vector, full-length human RXRα expression vector and/or the empty pcDNA3.1 vector together with 100 ng of reporter constructs containing the intact (−4282/+9 hAIV-LUC), site-directed mutant PPRE2 (−4282Mut/+9 hAIV-LUC) or deletion (−4282Δ/+9 hAIV-LUC) of the human apoA-IV promoter. *Renilla* luciferase plasmid pRL-TK (20 ng; Promega) was included in all transfections as an internal control for transfection efficiency. Forty-eight hours after transfection, cell extracts were examined using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity values were divided by those of *Renilla* luciferase activity to obtain normalised luciferase activities. Data are presented as the means ± S.E. of three independent experiments.

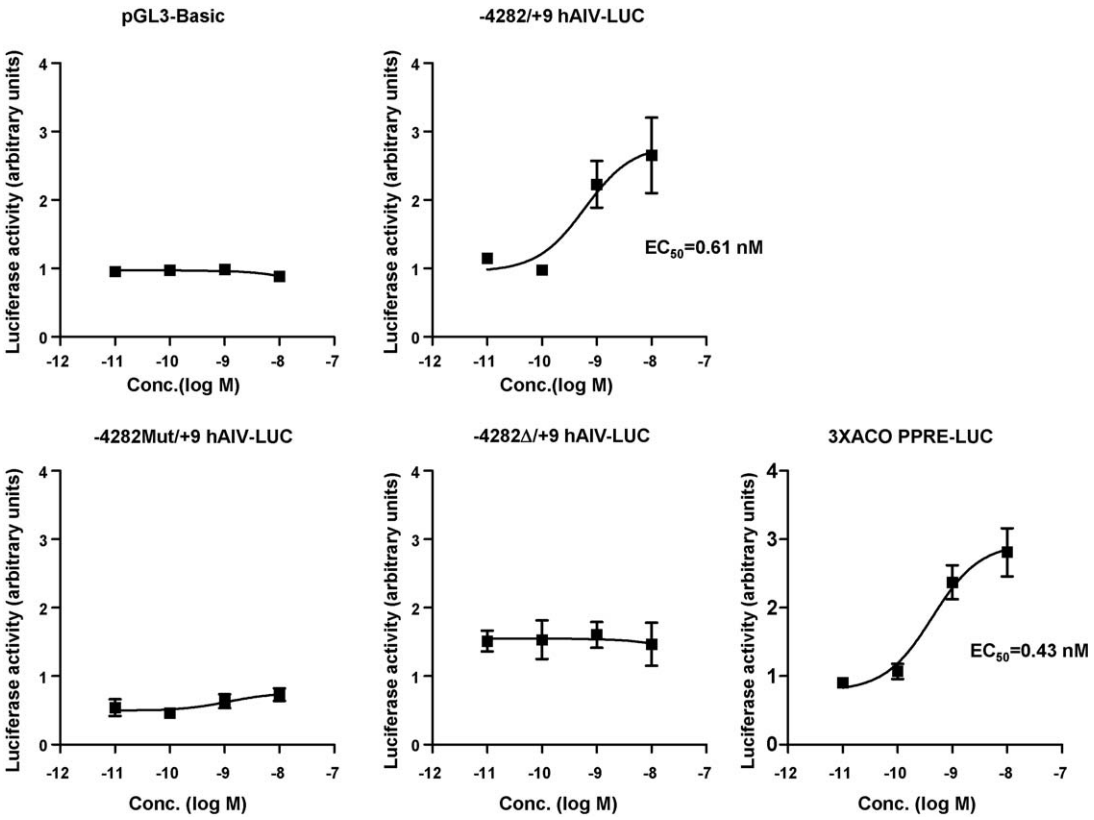

Fig. 6. Effects of KRP on transcriptional activation of the human apoA-IV promoter. HepG2 cells were co-transfected with 1 ng of the full-length human PPARα expression vector together with 100 ng of indicated reporter constructs. *Renilla* luciferase plasmid pRL-TK (20 ng; Promega) was included in all transfections as an internal control for transfection efficiency. After transfection for 2 h, cells were treated with the indicated concentrations of KRP for 48 h and cell extracts were examined using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity values were divided by those of *Renilla* luciferase activity to obtain normalised luciferase activities. The EC_{50} values of the compounds tested were derived by curve-fitting using the Prism program (GraphPad Software). Data are presented as the means ± S.E. of three independent experiments.

exogenously expressed PPARα in HepG2 cells (Fig. 6). KRP, a known agonist of human PPARα [11,32], increased luciferase activity (EC_{50}: 0.61 nM) in −4282/+9 hAIV-LUC-transfected cells, but not in those transfected with −4282Mut/+9 hAIV-LUC or −4282Δ/+9 hAIV-LUC (Fig. 6). These results indicated that the PPRE2, −2979/−2967 apoA-IV promoter is a major site for transactivation of human apoA-IV by PPARα agonist. Another PPARα agonist, fenofibric acid, also increased luciferase activity in a dose-dependent manner (100 μM, 2.03-fold; 300 μM, 3.78-fold, relative to vehicle control) in −4282/+9 hAIV-LUC-transfected cells, suggesting that a common regulatory site for transactivation by PPARα agonists is located in the −4282/+9 apoA-IV promoter. KRP increased luciferase activity (EC_{50}: 0.43 nM) in 3xACO PPRE-LUC-transfected cells (Fig. 6), indicating that KRP induces the transactivation of a typical PPRE as a PPARα agonist, and proving that this transient transfection assay is a suitable for evaluation of the agonist-induced transactivation.

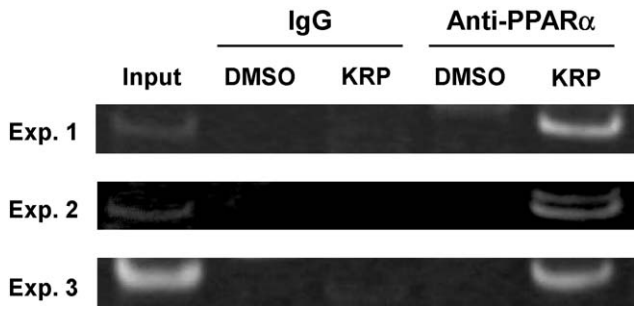

Fig. 7. ChIP assays on HepG2 cells. Cells were transfected with 300 ng of the full-length human PPARα expression vector. Twenty hours after transfection, cells were treated with 0.1% DMSO or 10^{-8} M KRP for 30 min, then fixed in fresh medium containing 1% formaldehyde for 10 min. Immunoprecipitation was performed using an antibody against PPARα or with IgG as a negative control. PCR products were visualised on ethidium bromide-stained 8% polyacrylamide gels. For comparison, amplification derived from unprecipitated chromatin is also shown (Input). The results of three independent experiments are shown.

3.5. Binding of PPARα to endogenous PPRE2 (−2979/−2967) in HepG2 cells

Finally, we investigated whether PPARα binds to endogenous PPRE2 in HepG2 cells by ChIP assay. A specific DNA fragment corresponding to PPRE2 was immunoprecipitated by anti-PPARα antibody in the presence of KRP (Fig. 7). No PPRE2-specific DNA was detected when rabbit preimmune IgG was used as a control (Fig. 7). These results indicate that PPARα binds to endogenous PPRE2, −2979/−2967 apoA-IV promoter in the presence of PPARα agonist.

4. Discussion

In this study, the human apoA-IV promoter was analysed to identify the PPARα-dependent regulatory region and to explore the mechanism of PPARα agonist-induced transactivation.

First, we searched for the region responsible for transcriptional regulation by PPARα/RXRα overexpression in the human apoA-IV promoter by transient transfection assay. Overexpression of PPARα/RXRα is known to induce transactivation in promoters containing PPRE in the absence of ligand [25]. Therefore, as the first

step, we searched for a functional PPRE induced by PPARα/RXRα overexpression. This assay revealed that −3279/−2261 is a key region for transactivation of the apoA-IV promoter by PPARα/RXRα (Fig. 1). Second, we focused on three putative PPREs in the −3279/−2261 apoA-IV promoter by sequence analysis, and identified a functional PPRE located at −2979/−2967 in the human apoA-IV promoter by EMSA (Figs. 3 and 4) and transfection assay (Fig. 5). The PPRE (−2979/−2967) consists of a perfect 5′-motif (TGACCT) and an imperfect 3′-motif (TGCTCC; 3 nucleotide differences) with 3 out of 7 matches in flanking region (Table 1) that sufficiently recruited PPARα [26]. This sequence is not fully canonical as a conserved PPRE; however, 34 nucleotides including −2979/−2967 bound strongly to PPARα/RXRα in EMSA (Fig. 3). We also showed that a single mutation in the perfect 5′-motif abolished PPARα/RXRα binding in EMSA (Fig. 4), and decreased transactivation by PPARα/RXRα overexpression in −4282/+9 hAIV-LUC-transfected cells (Fig. 5). Taken together, these results suggest that the identified nucleotide motif (−2979/−2967; TGACCTtTGCTCC) can serve as a PPRE in the context of the human apoA-IV promoter.

Next, we investigated whether PPRE (−2979/−2967) is a major site for PPARα agonist-induced transactivation. It has been validated that the transient transfection assay in the presence of exogenously expressed PPARα and utilizing the endogenous co-factors is suitable for evaluation of agonist-induced transactivation of PPARα-regulated genes [31]. KRP increased luciferase activity in typical 3xACO PPRE-LUC-transfected cells, indicated that this transfection assay can detect PPARα agonist-induced transactivation. We also evaluated the ligand-induced transactivation in exogenously both PPARα/RXRα co-expressed cells. The EC_{50} value of KRP was 0.21 nM and almost consistent with only expressed PPARα (EC_{50}: 0.43 nM, Fig. 6) in 3xACO PPRE-LUC-transfected cells. However, the fold induction levels of KRP-induced luciferase activity in PPARα/RXRα co-expressed cells was 2-fold less than only expressed PPARα (data not shown) because of the increase of background (luciferase activity) by PPARα/RXRα co-expression itself without PPARα agonist. Therefore, in this study, we selected the validated assay [23,31] that only exogenously PPARα expressed cells, and evaluated the importance of PPRE (−2979/−2967) in agonist-induced activation of the apoA-IV promoter by transfection assay. Consistent with the results of PPARα/RXRα overexpression, KRP-induced transactivation in −4282/+9 hAIV-LUC-transfected cells, but not in those transfected with −4282Mut/+9 hAIV-LUC or −4282Δ/+9 hAIV-LUC (Fig. 6). Fenofibric acid was also shown to increase luciferase activity in a concentration-dependent manner in −4282/+9 hAIV-LUC-transfected cells. These results indicated that either overexpression of PPARα/RXRα or activation of PPARα by agonist induces transactivation of apoA-IV promoter *via* PPRE (−2979/−2967).

Finally, ChIP assays were performed to determine whether KRP induces binding of PPARα to endogenous PPRE (−2979/−2967) in HepG2 cells. Fig. 7 shows that KRP markedly induced PPARα/PPRE complex in the native state. There was no band in DMSO controls, suggesting that binding of PPARα to endogenous PPRE (−2979/−2967) is PPARα agonist-dependent in the presence of exogenously expressed PPARα. In this assay, we used culture medium containing 10% delipidated foetal calf serum to decrease the background, and therefore almost no bands were expected in DMSO controls.

The results of this study indicated that a functional PPRE is located at −2979/−2967 in the human apoA-IV promoter and its PPRE specifically mediates the up-regulation of apoA-IV transcription by PPARα. It has been reported that apparent species difference was observed in regulation of apoA-IV gene in response to PPARα agonist between human/dog [11] and rodent [33,34]. To clarify the mechanism of species difference observed in apoA-IV regulation, further study will be needed to investigate the promoter region of rodent apoA-IV.

This paper presents the first evidence that apoA-IV is regulated directly by PPARα in human cells. Previous reports suggested that apoA-IV has physiological roles, including lipoprotein metabolism [16–19] and appetite in response to fat in the diet [20,21]. Most recently, apoA-IV possesses anti-atherogenic properties [16,22], and there is also evidence supporting apoA-IV anti-oxidant [22,35] and anti-inflammatory activity [36,37]. Therefore, up-regulation of apoA-IV induced by PPARα agonism in human may act as the beneficial therapy for atherosclerosis as well as dislipidaemia, and suggests the pharmacological significance of apoA-IV as a biomarker in PPARα agonist therapy for human subjects with dyslipidaemia.

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