PPARδ increases expression of the human apolipoprotein A-II gene in human liver cells

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Abstract. The peroxisome proliferator-activated receptor δ $(PPAR\delta)$ is a transcription factor that regulates genes of importance in lipid and glucose metabolism. ApoA-II is one of the major proteins of the HDL-particle. The aim of this study was to investigate the regulation of apoA-II gene expression by PPAR_δ. Treatment of HepG2 cells with the PPAR8 specific agonist GW501516 increased apoA-II mRNA expression. Likewise, reporter gene assays using a construct containing 2.7 kb of the proximal apoA-II promoter showed increased activity after treatment with GW501516, both in HepG2 and in HuH-7 cells. Mutation of two putative PPAR response elements (PPREs) in this region showed that the PPRE at position -737/-717 is the functional site. Binding of PPAR8 to this site was confirmed by chromatin immunoprecipitation and gel retardation analyses. In conclusion, PPARo increases the expression of the human apoA-II gene in liver cells via a PPRE in the proximal promoter.

Introduction

High-density lipoproteins (HDL) are inversely correlated with the incidence of coronary artery disease (CAD) in humans and have postulated roles in the atherosclerotic process such as reverse cholesterol transport, anti-inflammation and anti-oxidation (1). The main apolipoproteins of HDLs are apolipoprotein A-I (apoA-I) and A-II (apoA-II). The antiatherogenicity of apoA-I is well supported in experimental and clinical studies while the role of apoA-II in the atherosclerotic process, is less clear (2). However, there is recent data showing that apoA-II is associated with a decreased risk of cardio-vascular disease (3).

Fibrates increase plasma HDL cholesterol levels and have been shown to increase the levels of both apoA-I and apoA-II in plasma, thus supporting a positive correlation between apoA-II expression and plasma HDL cholesterol. Fibrates are activators of peroxisome proliferator-activated receptor α (PPAR α), a member of the PPAR family of ligand activated transcription factors. PPARs regulate lipid and glucose homeostasis and their natural ligands are fatty acids and fatty acid metabolites (4). PPARs regulate transcription by forming heterodimers with the retinoid X receptor (RXR) and binding to PPAR response elements (PPREs) in the promoters of their target genes. The PPRE consists of a direct repeat (DR) of the consensus sequence AGGTCA separated by one nucleotide, a DR1 motif. In addition, seven nucleotides in the 5'-flank of DR1 have been described to be of importance for binding to the PPRE (5). Studies regarding the effect of PPAR α activation using approximately 1 kb of the promoter of the ApoA-II gene, resulted in the identification of a functional DR1 in a regulatory region, designated as the J-element, positioned 716-734 bp upstream of the transcriptional start site (6,7).

PPAR δ is another member of the PPAR family, which has encountered a lot of attention in recent years partly due to its beneficial effects on glucose and lipoprotein metabolism in primates. Treatment of obese rhesus monkeys with a selective PPAR δ agonist, GW501516, increased plasma HDL cholesterol levels by 40% and both apoA-I and apoA-II levels were elevated (8). Further, subjects obtaining GW501516 in a phase I clinical trial showed increased HDL cholesterol levels compared to placebo (9). Thus, GW501516 is a candidate drug for treatment of metabolic disorders (10).

We hypothesize that activation of PPARô increases apoA-II expression via a direct transcriptional mechanism, which might contribute to the HDL increase in plasma cholesterol levels seen in primates treated with GW501516. In this study, we aimed to identify and characterize the functional PPREs located within the proximal promoter of the human apoA-II gene.

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Materials and methods

Chemicals. GW501516 was synthesized by Synthelec AB, Sweden as described (11). Fenofibric acid (FF) was a gift from professor Per Eriksson (Karolinska Institutet, Sweden).

Cell culture. The human hepatoma cells, HuH-7, were kindly provided by Dr John McLauchlan (University of Glasgow, UK) and the human liver cell line, HepG2, was purchased from ATCC (Rockville, MD, USA). The cells were cultured in DMEM (1 g/ml glucose, Invitrogen, USA), containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C in 5% CO₂. For Taqman assays the cell medium contained 10% lipid deficient FBS, which was depleted of lipids and hormones by treatment with fumed silica (s5130, Sigma-Aldrich, USA), AG-1-X8 resin (Bio-Rad, USA) and activated dextran-coated charcoal (C6197, Sigma-Aldrich).

RNA isolation, cDNA synthesis and real-time PCR. Cells were seeded out in 6-well plates in 10% lipid deficient DMEM at a density of 1x10⁶ cells/well, incubated for 24 h at 37°C followed by treatment with GW501516, FF or vehicle (DMSO) for 48 h. Medium containing fresh agonists was added after 24 h. Total RNA was extracted, reverse transcribed and the cDNA was amplified by real-time PCR as described (14) using apoA-II primers (5'-cagacaccaaggacagaga, 5'-cagatggtg aggagtagca) and probe (5'-ctggctaggccgccctccc) (Thermo Electron, Germany). RPLPO was used as a house-keeping gene; with the primers (5'-agcaagtgggaaggtgtaatcc, 5'-ccattcta tcatcaacgggtacaa) and probe (5'-tctccacagacaaggccaggac tcgt).

In silico analyses. Approximately 3 kb of the apoA-II promoter (GenBank accession no. AL590714) was analysed for putative PPREs using the MatInspector program at the Genomatix web site (12). Mulan was used for the sequence comparisons between species (13).

Cloning. The promoter region of apoA-II was cloned by long-range amplification (Roche, USA) according to the manufacturer's instructions using human genomic DNA as template. The primers 5'-tggaacaggaagagtagggaa (-2725 to -2704) and 5'-ctcacacatcttgcctctta (+40 to +61) were used for amplification (Proligo, France). The PCR product was subcloned using the TA-cloning kit (Invitrogen) according to the manufacturer's instructions. A *XhoI* and *KpnI* fragment of the apoA-II construct was subsequently transferred to the pGL3-basic vector (Promega, USA). DNA sequencing was performed using Big Dye Terminator Kit and an automatic sequencer (Genetic Analyzer 3100, Applied Biosystems) to confirm that the sequence was in accordance with the genomic DNA sequence.

Transient transfection assays. Plasmids were purified using the Endofree Plasmid Maxi kit (Qiagen, UK). Transfections were performed as described (11,14) and then incubated with GW501516 at different concentrations in DMEM (0.2% FBS) for 24 h. Subsequently, the cells were harvested and luciferase activity was measured as described (11). Mean values of four replicates for each treatment were calculated.

In vitro mutagenesis. Site-directed mutagenesis of the PPREs was accomplished using a QuickChange Site-directed mutagenesis kit (Stratagene, USA) and mutagenic primers (Proligo, France) corresponding to the O- and J-site, respectively (O-site: 5'-ggtgacaagtggGGAGGAgAGATTGaagatggggggg, J-site: 5'-ggcttctaccAGAGTAaAGCTTGaaggcacc), according to the manufacturer's instructions. The underlined nucleotides denote mutated sites.

Coupled transcription/translation. The human PPAR δ sequence was cloned into a TNT vector as described (20). TNT-hPPAR α and TNT-hRXR α were kind gifts from Ingalill Rafter (AstraZeneca, Sweden). *In vitro* transcription/translation was performed using the rabbit reticulocyte lysate system according to manufacturer's advice (Promega) as described (14).

Electrophoretic mobility shift (EMSA). A double stranded oligonucleotide containing the J-site spanning -740 to -714 of the human apoA-II promoter (J-Wt: 5'-cttctaccAGGGTAaA GGTTGaaggcac) and an identical oligonucleotide containing two point mutations (J-Mt: 5'-cttctaccAG<u>A</u>GTAaAG<u>C</u>TTG aaggcac) were used for EMSA studies. The rat ACO-PPRE was used as a control (5'-ggaccAGGACAaAGGTCAcgttcgg). EMSA assays were conducted as described (14). In short, 2 μ l of *in vitro* produced PPAR α or PPAR δ together with 2 μ l of RXR α or mock lysate (empty vector), was used in each assay. For competition analyses, a 100-fold molar excess of unlabelled double stranded J-Wt, J-Mt or ACO-oligo was added. DNA-protein complexes were run on a 6% polyacrylamide gel and analysed as described (14).

Chromatin immunoprecipitation (ChIP) assay. HepG2 cells were treated with 100 nM of GW501516 for 8 h and were subsequently fixed using formaldehyde. Chromatin was purified and sheared using an enzymatic ChIP-IT express kit according to the manufacturer's instructions (Active Motif, USA), followed by immunoprecipitation with a PPAR_δ antibody (sc-7197, Santa Cruz, USA) and an IgG from a ChIP-IT human control kit (Active Motif). Immunoprecipitated DNA was subjected to 36 cycles of PCR with one primer pair amplifying the apoA-II J-site region (apoA-II_J-siteF: 5'-cact gcctagaactgataagg, apoA-II_J-siteR: 5'-ctggatgctgatcactgaaca) and a second pair of control primers amplifying a region 2.5 kb downstream of the J-site (apoA-II_negF: 5'-tgaatccctcgatttctg gag, apoA-II_negR: 5'-cccggttcaaaagattctcatg) generating a 177 or 253 bp product, respectively. Chromatin input (10%) was used as positive while H₂O was used as blank control templates for both primer pairs.

Statistical analyses. In order to establish differences in promoter activity or mRNA expression between control and treated cells, Student's t-tests (two-tailed) were used. Significant differences are indicated as: *p<0.05, **p<0.01, ***p<0.001.

Results

GW501516 increases apoA-II gene expression in human hepatoma cells. To analyse whether activation of PPARð regulates apoA-II gene expression in human liver cells,



Figure 1. GW501516 (GW) (100 nM) and fenofibric acid (FF) (500 μ M) induce apoA-II mRNA expression in HepG2 cells after 48 h of treatment. Results are shown as relative expression compared to cells treated only with vehicle (n=4). Significant differences: *p<0.05, ***p<0.001.

HepG2 cells were treated with GW501516 followed by RNA isolation and cDNA analysis. As shown in Fig. 1, addition of GW501516 (100 nM) under present conditions, increased apoA-II mRNA levels by 1.22 ± 0.09 fold, p<0.05. Treatment with 500 μ M fenofibric acid (FF) was used as a positive control (1.56±0.07, p<0.001).

In silico analysis of the apoA-II proximal promoter region reveals two putative PPREs. In order to investigate whether the increase in expression of apoA-II mRNA by PPAR8 agonist treatment could be due to the presence of PPREs in the promoter region, in silico analyses of the apoA-II promoter region was performed. Two putative PPREs located at -2656 to -2636 and -737 to -717, respectively, relative to the 5'-cap site were identified and hereafter designated as the O- and J-site. Examination of these PPREs revealed that both the O- and J-sites contain 9 out of 13 nucleotides of the core DR1 consensus. Analysis of the 5'-flanking sequence showed that the O-site contains 4 out of 7 nucleotides, while the J-site contains 2 out of 7 nucleotides compared to the consensus sequence (Fig. 2A). In addition, both putative PPREs are conserved in closely related species such as the macaque and chimpanzee. In mice, the DR1 element of the O-site deviates two bases compared with the human sequence showing 92% homology, whereas the J-site is disrupted by 15 nucleotides (Fig. 2B).

Treatment with PPAR δ agonist increases apoA-II promoter activity. The proximal promoter of the apoA-II gene encompassing both putative PPREs was cloned into a reporter vector to study whether PPAR δ induces transcriptional activity. The construct containing 2.7 kb of the human apoA-II promoter was transiently transfected into two hepatoma cell lines, HepG2 and HuH-7, which were subsequently treated with various doses of GW501516.

In HepG2 cells (Fig. 3A), treatment with 10 nM of GW501516 did not show any significant increase in promoter activity unless a PPAR δ expression vector was cotransfected, suggesting low endogenous expression of PPAR δ in these cells (1.71±0.93, p>0.05). Accordingly, cotransfection with a PPAR δ expression vector without any additional treatment increased basal promoter activity up to 6-fold (6.02±0.35, p<0.01), whereas treatment with GW501516 in the presence

A Consensus		<u>5'-flank</u> DR1 caaaa ^a ctaggtca ^a aggtca	
	O-site	caagtggGGGTGAgAGATCA	4/7 + 9/13
	J-site	ttctaccAGGGTAaAGGTTG	2/7 + 9/13
в			
O-site	Human Pan Tr. Macaque Mouse	caagtggGGGTGAgAGATCA caagtggGGGTGAgAGATCA caggtggGGGTGAgAGATCA caggtggGGGTGAgGGATCA ** ************	
J-site	Human Pan Tr. Macaque Mouse	ttctaccAGGGT ttctaccAGGGT ttctgccAGGGT tctcatcAATGTGGGTCCCCCC * ** **	AAAGGTTG AAAGGTTG AAAGGTTG GCCTCCAAAAAACA ***

Figure 2. Comparison of putative PPREs. The DR1 nucleotides are given in capital letters while the nucleotides of the 5'-flanking region are shown in lower-case letters. (A) Comparison of identified PPREs in the apoA-II promoter designated as the O-site and J-site, respectively, and the consensus PPRE sequence. The number of matches for the 5'-flanking region and the core DR1, respectively, compared to the consensus sequence are shown to the right. Identical nucleotides between the consensus sequence and the PPREs are denoted by an asterisk. (B) Alignment of genomic sequences in the region of the apoA-II O- and J-sites between human, pan troglodytes (chimpanzee), maqaque and mouse. Identical nucleotides are highlighted by an asterisk.

of PPAR δ expression increased the promoter activity by 20-fold (20.69±0.34, p=0.001). In HuH-7 cells (Fig. 3B), the apoA-II promoter activity increased already at 10 nM GW501516 (1.37±0.10, p<0.05). Cotransfection with PPAR δ alone did not affect the promoter activity significantly (1.49±0.26, p>0.05), but together with GW501516, the apoA-II promoter was induced almost 4-fold (3.50±0.32). Treatment with 200 μ M FF or cotransfection with PPAR α alone increased the apoA-II promoter activity in both cell lines (data not shown).

ApoA-II regulation by PPAR δ is mediated via the J-site. In order to investigate the functional effects of the two putative PPREs in the apoA-II promoter, in vitro mutagenesis of these two sites were performed followed by transfection in HuH-7 cells. The transcriptional activities of the mutated constructs were compared with the activity of the wild-type construct (Fig. 3C). Mutation of both putative PPREs resulted in a loss of induction by PPAR δ . This was also noted for PPAR α (data not shown). However, mutation of the O-site alone did not affect the apoA-II promoter activity after PPAR activation compared to the wild-type construct, whereas mutation of the J-site ablated PPAR δ and PPAR α activation of the apoA-II promoter. These results indicate that the J-site is the functional PPRE in the promoter region for both PPAR δ and PPAR α . In addition, basal luciferase levels of the constructs containing a mutated J-site (Mt-J, Mt-OJ) were suppressed compared to the activities of the constructs containing either the wild-type (Wt) or the mutated O-site (Mt-O).

PPAR δ binds to the apoA-II J-site in vitro and in vivo. In order to investigate whether PPAR δ and PPAR α can bind to

GW

PPARS



Figure 4. Binding of PPARo to the apoA-II J-site. (A) EMSA showing binding of PPARa:RXRa and PPARo:RXRa heterodimers to the wild-type (W) but not to the mutated (M) J-site. Labelled wild-type or mutated J-site specific oligonucleotides were incubated with in vitro synthesized human PPARs and RXRa. Competition assay was performed by adding 100-fold excess of cold wild-type (W), mutant (M) or ACO-PPRE (A) probes. Arrows indicate the positions of the PPAR α :RXR and PPAR δ :RXR DNA:protein complexes, respectively. (B) HepG2 cells were treated with GW501516 (100 nM) for 8 h, fixed and chromatin was purified followed by enzymatic digestion. ChIPs were performed using a PPAR δ antibody and negative control IgG. Immunoprecipitated DNA was subjected to PCR with a primer pair specific to the apoA-II J-site (lanes 1-4). As a negative control a second set of primers was used to amplify a region 2.5 kb downstream of the J-site (apoA-II neg, lanes 5-8). PCR was performed on DNA isolated through ChIP using chromatin input as a positive control (lanes 1 and 5), negative control IgG (lanes 2 and 6), PPARo antibody (lanes 3 and 7) and blank H₂O (lanes 4 and 8).

Figure 3. Effects of GW501516 and/or PPARδ overexpression on apoA-II promoter activity in (A) HepG2 and (B) HuH-7 cells. The luciferase activity of transfected untreated control cells in each experiment was set to one and the resulting activities for the test agents were calculated relatively to the control cells (n=4). (C) Transfection assays in HuH-7 cells using constructs with mutated PPREs in the presence or absence of PPARδ overexpression together with GW501516 treatment (10 nM). Black bars show promoter activity in cells transfected with the wild-type (Wt) or mutated (Mt-O, Mt-J or Mt-OJ) constructs in the absence of PPARδ overexpression or GW501516. Grey bars denote luciferase activity in cells transfected with wild-type or mutated constructs and PPARδ expression plasmid, which were subsequently treated with GW501516 (10 nM) for 24 h (n=4). Significant differences compared to untreated controls: *p<0.05, **p<0.01, ***p<0.001.

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the J-site located at -737 to -717 in the apoA-II promoter, electrophoretic mobility shift assays (EMSA) were performed. Human PPAR δ /RXR α as well as PPAR α /RXR α heterodimers resulted in the formation of retarded complexes, which were not detected using RXR α , PPAR δ or PPAR α alone (Fig. 4A). No binding was observed when the J-site was mutated. Competition studies using unlabelled apoA-II_J-PPRE or ACO-PPRE sequences diminished protein:DNA complex formation, whereas no change was seen when a mutated apoA-II_J-probe was used. To confirm that PPAR δ binds to the J-site also in vivo, a chromatin immunoprecipitation (ChIP) assay was performed. Immunoprecipitated chromatin DNA using a PPAR_δ antibody, generated a PCR-product with a primer pair flanking the J-site in the apoA-II promoter (Fig. 4B, lane 3) but not with a pair of negative control primers binding 2.5 kb downstream (Fig. 4B, lane 7). ChiP analysis using a negative control antibody did not give rise to any PCR-product with neither of the primer pairs (Fig. 4B, lanes 2 and 6). Thus, the ChIP assay confirmed binding of PPARδ to the J-site.

Discussion

PPARδ has received a great deal of attention during recent years since PPARδ activation has been shown to elevate plasma HDL and decrease triglyceride levels in primates. We previously performed a microarray cDNA analysis comparing HepG2 cells treated with the PPARδ specific ligand, GW501516, with untreated cells in order to identify PPARδ target genes involved in lipid metabolism. The data from this screening indicated that GW501516 treatment results in increased expression of apoA-II (unpublished data). Plasma apoA-II levels are controlled mainly by synthesis in the liver rather than catabolism (15) and is one of the major apolipoproteins of HDL recently assigned to possess beneficial properties in HDL cholesterol metabolism and atherosclerosis (3).

This study investigated the effects of PPAR^δ on apoA-II gene expression and was designed to identify functional PPREs involved in the transcriptional regulation. Although, the observed induction of the apoA-II gene expression by PPAR⁸ activation in this study seems modest, the regulation of the apolipoprotein genes in vitro has been shown to be highly dependent on the batch of serum used. Induction of apoA-II mRNA expression by the same concentration of fibrates has been reported to vary from 1.5- to 2.5-fold using different batches of serum, both within and between studies (7,16). In the present study, the mRNA expression of apoA-II increased by 1.5-fold after treatment with fibrates, indicating that the batch of serum used may contribute to the overall moderate effect on apoA-II mRNA expression. Thus, the magnitude of induction might not be as important as the reproducibility of the activation itself.

The results from reporter gene assays in this study, where PPAR δ activation resulted in a 4-fold increase in promoter activity using HuH-7 cells and as much as a 20-fold increase using HepG2 cells, are further evidence of a PPAR δ inducing effect on the apoA-II promoter *in vitro*. Accordingly, obese rhesus monkeys treated with GW501516 show plasma apoA-II elevations of approximately 21%, indicating that apoA-II expression is induced by PPAR δ activation also *in vivo* (8).

The results of this study show that the effect of PPAR δ activation on the human apoA-II promoter is mediated via a PPRE located at -737 to -717, upstream of the cap site, which corresponds to the regulatory J-site as determined by DNase I footprinting analyses (6). The putative PPRE in the apoA-II promoter positioned at -2656 to -2636 relative to the cap site, the O-site, has not been analysed before. Although, this site is conserved between several species and shows a high degree of homology to the PPRE consensus sequence, it does not seem to play a role in the transcriptional regulation of the human apoA-II gene by PPARs. One of the reasons might be that this site is located within an intron of the TOMM40L gene. The apoA-II and TOMM40L genes are organised in a head-to-head fashion and only 2414 bp separates the transcriptional start sites of the respective genes. The function of TOMM40L is as yet unknown but studies have shown that this gene is transcribed (17).

The J-site is the same functional PPRE that has been shown to mediate the effects of PPAR α activation (7), which is not surprising since all the PPARs are reported to share a common consensus binding site on the DNA. However, it has been reported that the 5'-flanking region of the PPRE is of importance for PPAR isotype binding of DNA and that some PPARs can bind to imperfect PPRE sequences whereas others cannot, intriguingly suggesting distinct roles for different PPAR family members (5). The J-site constitutes an important regulatory region in the apoA-II promoter, as these data suggest, since the basal transcriptional activity decreased when the site was mutated, which is in agreement with a previous study (7). Of note, the J-site has been shown to be important not only for mediating the effects of PPARs but also for binding of hepatocyte nuclear factor- 4α and other members of the nuclear receptor family (18). In addition, others have shown that the region located -911 to -614 encompassing the J-site is required for basal transcription from the apoA-II promoter (6).

In conclusion, we have shown that the human apoA-II gene is transactivated by PPAR δ through binding to the J-site in the promoter. Since increased synthesis of apoA-II is correlated with elevated plasma HDL cholesterol concentrations, apoA-II might be one of many genes responsible for the beneficial effects regarding lipid and lipoprotein metabolism observed as a result of PPAR δ activation.

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