Apolipoprotein A5, a Crucial Determinant of Plasma Triglyceride Levels, Is Highly Responsive to Peroxisome Proliferator-activated Receptor α Activators*

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The recently discovered APOA5 gene has been shown in humans and mice to be important in determining plasma triglyceride levels, a major cardiovascular disease risk factor. apoAV represents the first described apolipoprotein where overexpression lowers triglyceride levels. Since fibrates represent a commonly used therapy for lowering plasma triglycerides in humans, we investigated their ability to modulate APOA5 gene expression and consequently influence plasma triglyceride levels. Human primary hepatocytes treated with Wy 14,643 or fenofibrate displayed a strong induction of APOA5 mRNA. Deletion and mutagenesis analyses of the proximal APOA5 promoter firmly demonstrate the presence of a functional peroxisome proliferator-activated receptor response element. These findings demonstrate that APOA5 is a highly responsive peroxisome proliferator-activated receptor α target gene and support its role as a major mediator for how fibrates reduce plasma triglycerides in humans.

Coronary heart disease continues to be a major cause of morbidity and mortality worldwide. Several epidemiological studies established that, in addition to elevated low density lipoprotein and reduced high density lipoprotein level, elevated triglycerides (TGs) constitute an independent risk factor for coronary heart disease (1, 2). In addition, hypertriglycerideremia is often associated with the metabolic syndrome that characterizes diabetes and obesity (3, 4). Therefore, the identification of factors or genes affecting triglyceride metabolism is of significant medical importance for the correction of hypertriglyceridermia and associated coronary heart disease.

Apolipoproteins play a determinant role in lipoprotein metabolism and in lipid homeostasis. More specifically, the APOA1/C3/A4 apolipoprotein gene cluster is tightly linked to plasma lipid profiles. Indeed, mutations in members of this cluster have been shown to cause severe dyslipidemia and heightened atherosclerosis susceptibility (5–8). A comparative genomic characterization of the APOAI/C3/A4 gene cluster flanking regions led to the recent identification of a new apolipoprotein gene, apolipoprotein A5 (APOA5), present in both mice and humans (9). apoAV shares homology with several apolipoproteins, most prominently apoAIV, and is 368 and 366 amino acids long in mice and human, respectively. This gene appears to be predominantly expressed in the liver and resides on high density lipoprotein and very low density lipoprotein particles (9, 10).

The properties of apoAV were investigated by creating human APOA5 transgenic and knock-out mice and by searching for associations between human APOA5 polymorphisms and plasma lipid parameters. The ApoA5 knock-out mice display a 400% increase in plasma triglycerides compared with wild-type mice, while APOA5 transgenics exhibit triglyceride levels corresponding to one-third of those in control mice. This determinant link between APOA5 and triglycerides was supported in several separate human studies through the consistent demonstration of associations between APOA5 single nucleotide polymorphisms and plasma triglyceride levels (9, 11–13). Taken together these mouse and human studies highlight the importance of APOA5 in the determination of triglyceride plasma levels, and the clinical relevance of identifying factors regulating APOA5 expression.

Fibrates are hypolipidemic drugs with pleiotropic effects on lipid metabolism including the reduction of plasma triglycerides. Classically, the triglyceride lowering action of fibrates is explained by decreased hepatic secretion of very low density lipoprotein and an enhancement in plasma triglyceride clearance. Several studies established that this effect is mediated through the induction of lipoprotein lipase expression (14) and down-regulation of APOC3 expression by fibrates (15). A major means by which fibrates regulate the expression of lipid metabolism-related genes has been shown to be via activation of the peroxisome proliferator-activated receptor α (PPARα) (16). Three distinct PPARs (α, β, and γ) have been described in different species (17). PPARs are ligand-activated nuclear receptors that dimerize with the retinoid X receptor (RXR) and bind to specific DNA sequence defined as peroxisome proliferator response elements (PPREs). Upon binding, PPARs activate gene transcription.

Given the determinant link between APOA5 and plasma triglycerides and the widespread use of fibrates in the treatment of dyslipidemia, we investigated whether fibrates can modulate APOA5 gene expression and consequently influence plasma triglyceride levels. Our studies with human hepatocytes revealed that fibrates dramatically increase APOA5 ex-
pression. Coupled with in vitro promoter analysis and the demonstration of a functional APOA5 PPRE, these data identify fibrates acting via PPARα as a crucial regulator of the new apolipoprotein APOA5 and suggest a novel clinical mechanism for how PPARα activators can influence triglyceride homeostasis.

**EXPERIMENTAL PROCEDURES**

**Cloning and Construction of Recombinant Plasmids**—Exon 1 of the human and mouse APOA5 gene were determined by examination of the expressed sequence tag data base and identification of numerous cDNA clones that terminate at a common 5′ base pair in the genome sequence. The four exon structure of APOA5 is consistent with that of the evolutionarily related apolipoprotein genes (APOA1, APOC3, and APOE). Human APOA5 promoter fragments (−846/+62, −305/+62, −147/+62) were amplified by PCR using a APOA5 genomic BAC clone (9) as template and cloned in pGL3 luciferase vector. The followed forward oligonucleotides 5′-AGACCTGTGGAGGCTATGAGTCATGGGA-3′ and reverse oligonucleotide 5′-AAGGTCATGGGA-3′ were used for the PCR reaction. Site-directed mutagenesis (Stratagene) of the APOA5 promoter was accomplished using the oligonucleotide 5′-AGTGGGAGCACTAGTGGGTT-3′ as a mutagenic primer. The human APOA5-PPRE (5′-GATCCGGGAAGGTTAAGACCTGTTG-3′) site oligonucleotide was cloned in tandem repeat into BglII/HindIII sites of pC2OH, digested with HindIII, and subcloned upstream of the thymidine kinase (TK) promoter of pBLCAT4 as described previously (18).

**Cell Culture and RNA Analysis**—Primary human hepatocytes were isolated, maintained, and treated with fibrates as described previously (18). The 0.9-kb APOA5 cDNA probe was amplified by PCR with full-length APOA5 cDNA as template and the oligonucleotides 5′-GATAATGGCAAGCATGGCTG-3′ and reverse oligonucleotide 5′-CTGCAGGTAGTGTCTCCGCTGCGCCA-3′ and subcloned in plB-SK +.

**Transfections and Transient Expression Assay**—Human hepatoma HepG2 cells were maintained and transiently transfected by calcium phosphate coprecipitation as described (18) using 0.3 μg of reporter vector, 30 ng of PPARα expression vector, and 30 ng of CMV-β-galactosidase expression vector as a control for transfection efficiency.

**Gel Retardation Assay**—mPPARα and mRXRα proteins were synthesized in vitro using the rabbit reticulocyte lysate systems (Promega). Gel retardation assays were performed as described previously (18).

**RESULTS**

**Human APOA5 Gene Expression Is Induced by Fibrate Treatment in Human Primary Hepatocytes**—To determine whether fibrates can modulate APOA5 gene expression in humans, we analyzed APOA5 mRNA levels in primary human hepatocyte upon treatment with fenofibric acid, the active form of fenofibrate, or Wy 14,643, a prototype PPARα agonist. Treatment with fenofibric acid at a concentration (100 μM) similar to that reached in plasma from treated patients dramatically induced APOA5 mRNA levels (about 5-fold increase). We observed a similar effect with Wy 14,643 treatment (Fig. 1). These observations demonstrate that fibrates induce the expression of human APOA5, suggesting APOA5 as a new target gene for fibrates.

**Gene Regulation of APOA5 by Fibrates Occurs at the Transcriptional Level**—To determine whether APOA5 was directly responsive to PPAR, we examined the proximal APOA5 promoter for potential PPRE sites (Fig. 2). Upstream of exon 1 a consensus TATAA and CAAT box were readily apparent, as was a putative PPRE site (−272/−260). To delineate the mechanism of regulation of APOA5 gene expression by fibrates, we performed functional analysis of the APOA5 promoter. HepG2 cells were transiently transfected with a luciferase reporter vector driven by different human APOA5 promoter fragments (from −846/+62, −305/+62, −147/+62). Cotransfection with PPARα strongly stimulated the APOA5 promoter activity (about a 25-fold increase with construct −846/+62 and about a 40-fold increase with construct −305/+62) in the presence of Wy 14,643 (Fig. 3). Transcriptional activity of the APOA5 reporter construct was also slightly induced by the addition of Wy 14,643 in the absence of cotransfected PPARα. Deletion analysis of the promoter located a putative PPRE between nucleotides −305 and −147 from the initiation start site. These results support that the gene regulation of APOA5 by fibrates occurs at the transcriptional level.

**APOA5 Contains a PPRE That Confers Responsiveness to PPARα**—Transcriptional activation of APOA5 gene by PPARα suggests the presence of a functional PPRE in the APOA5 promoter. Sequence analysis revealed the presence of a putative PPRE with a high degree of homology between APOA5-PPRE and the PPRE consensus defined for PPARs (19). To
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To assess whether the putative PPRE mediates the PPARα effect, we performed transfection experiments using −305/+62 promoter construct containing a mutated version of the PPRE. The mutation is designed to suppress the binding of PPAR as described previously (18). Mutation of the PPRE abolished the activation of the APOA5 promoter by PPARα (Fig. 4).

To prove that the putative APOA5-PPRE could function as a PPRE, we cloned it in three oriented copies in front of the heterologous promoter TK and challenged with PPARα in HepG2 cells. We found that this site could transmit PPARα activation (about 5.8-fold increase in the presence of PPARα and Wy 14,643) to the TK promoter (Fig. 5A). We performed electrophoretic mobility shift experiments to examine whether PPAR-RXR heterodimer could bind to the PPRE. Incubation of labeled PPRE oligonucleotide with in vitro translated PPARα and RXRs resulted in the formation of a strong retarded complex (Fig. 5B). Taken together these results demonstrate that APOA5 promoter contains a functional PPRE that confers PPARα activation responsiveness.

**DISCUSSION**

Fibrates are among the most effective agents for lowering plasma triglycerides in humans. In this report, we show that fibrates dramatically affect the expression of the recently identified APOA5 gene in humans. The previous studies demonstrating that apoAV is selectively produced by the liver and behaves as a regulator of plasma triglyceride levels (21), triglyceride metabolism.

The up-regulation of human APOA5 is important regarding of the molecular mechanism of triglycerides homeostasis action of fibrate. Indeed, APOA5 represents the first example of an apolipoprotein whose overexpression leads to a decrease in triglyceride levels (9, 20), whereas APOC3 or another apolipoprotein tranegene leads to an increase in triglycerides plasma levels (21–24).

The activation of APOA5 transcription may be attributed to a PPRE located inside the proximal APOA5 promoter. Remarkably, this PPRE differs 1 nucleotide from the consensus PPRE (AGGTCA) A (AGGTCA) or DR-1 (direct repeat 1). Binding and functional studies indicate that this PPRE confers a significant PPARα-mediated transactivation. The rate of transcriptional up-regulation induced by PPARα activation on APOA5 is among, if not the highest, level attained for genes regulated by fibrates studied in human primary hepatocyte model (APOA2 (18) and CPT-1 (25)).

Actually, gene regulation via PPREs is complex. Indeed, the DR-1 structure of the PPRE can integrate different antagonistic actions induced by nuclear receptors such as RXR, retinoic acid receptor (RAR) (26), HNF4, and ARP-1 (or COUP-TF) (27) depending on the sequence of the regulatory element and the context of the promoter. The APOC3 gene harbors one DR-1 that can bind PPAR/RXR heterodimer, but its promoter responds to PPAR only in non-hepatocyte cells (26), whereas HNF4 (28) or RXR induce transactivation (26). Fibrates down-regulate APOC3 gene expression probably through indirect inhibition of the HNF4 nuclear receptor (29). Alternatively, PPARα activators stimulate Rev-erba (30) that in turn represses APOC3 (31). Therefore, APOA5 is the first triglyceride lowering apolipoprotein gene that is firmly identified as a positive and a direct target gene of PPARα activators.

So far, most of the TG lowering effects have been mainly attributed 1) to the induction of lipoprotein lipase gene expression, which enhances catabolism of TG-rich particles (14), and 2) to the down-regulation of APOC3 gene (15), leading to a decreased hepatic very low density lipoprotein secretion. The exact contribution that alterations in expression of these genes have on plasma triglyceride levels is not known but appears to
be dependent on the activation by PPARα. Analysis of the APOA5 transgenic and knock-out mice showed that their changes in plasma triglyceride levels were directly opposite to those previously reported for the APOC3 knockout and transgenic mice (21, 22). The APOA5 knock-outs display a 400% increase in plasma TG compared with the 30% decrease observed in Apoc3 knock-outs, whereas APOA5 transgenic showed decreased triglyceride levels compared with the increase reported in APOC3 transgenics. In addition, overexpression of APOA5 was accompanied by a lowered apoc3 protein level (9). Therefore, APOA5 may be defined as a major determinant of triglyceride levels and from the present study appeared to be a fibrate responsive through action of PPARα. Based on the magnitude of the effect that altered APOA5 expression has on plasma triglycerides in mice compared with APOC3, APOA5 may be defined as a potentially major determinant of triglyceride homeostasis. The results of the present study convincingly demonstrate that APOA5 is a target gene for PPARα activators. This gene regulation of APOA5, combined with the dramatic effects previously shown in APOA5 transgenic and knock-out mice on plasma triglyceride levels, suggest a plausible explanation for the ability of PPARα activators to lower plasma TG levels. Modulation of APOA5 via a PPARα pathway offers a new strategy for intervention designed at correcting hypertriglyceridermia and at limiting TG-associated metabolic disease and cardiovascular risk.

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