Evidence That Peroxisome Proliferator–Activated Receptor Delta Influences Cholesterol Metabolism in Men

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Objective—The objective of this work was to explore the role of peroxisome proliferator–activated receptor delta (PPARD) in lipid metabolism in humans.

Methods and Results—PPARD is a nuclear receptor involved in lipid metabolism in primates and mice. We screened the 5’-region of the human gene for polymorphisms to be used as tools in association studies. Four polymorphisms were detected: −409C/T in the promoter region, +73C/T in exon 1, +255A/G in exon 3, and +294T/C in exon 4. The frequencies of the rare alleles were 4.2%, 4.2%, 1.2% and 15.6%, respectively, in a population-based group of 543 healthy men. Only the +294T/C polymorphism showed significant association with a metabolic trait. Homozygotes for the rare C allele had a higher plasma LDL–cholesterol concentration than homozygotes for the common T allele, which was verified in an independent cohort consisting of 282 healthy men. Transfection studies showed that the rare C allele had higher transcriptional activity than the common T allele. Electrophoretic mobility shift assays demonstrated that the +294T/C polymorphism influenced binding of Sp-1. An interaction with the PPAR alpha L162V polymorphism was also detected for several lipid parameters.

Conclusions—These findings suggest that PPARD plays a role in cholesterol metabolism in humans. (Arterioscler Thromb Vasc Biol. 2003;23:637-643.)

Key Words: genetics ■ cholesterol ■ peroxisome proliferator–activated receptor ■ polymorphism

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors involved in the regulation of lipid and glucose metabolism. Three distinct PPARs, termed alpha (PPARA), gamma (PPARG), and delta (PPARD), have been isolated. They are encoded by separate genes and characterized by distinct tissue and developmental distribution patterns. PPARD is, in contrast with the other two PPARs, ubiquitously expressed.1 PPARA is involved in lipid metabolism and fatty acid oxidation whereas PPARG influences adipocyte differentiation and insulin action.2 The function of PPARD is not yet fully understood, but it has been shown to play a role in cholesterol metabolism in animal models. Treatment with a PPARD agonist increased plasma cholesterol concentrations in mice with a dysfunctional leptin receptor (db/db mice).3 A recent study showed that treatment with a potent selective PPARD agonist increased plasma cholesterol concentrations, decreased plasma triglyceride concentrations, increased HDL cholesterol, and decreased the fraction of small and dense LDLs in obese rhesus monkeys.4 Furthermore, PPARD plays a central role in fatty acid–controlled differentiation of preadipocytes, where it promotes induction of PPARG and other genes involved in adipocyte differentiation.5,6 Taken together, these data suggest that PPARD may play a role in the development of metabolic perturbations associated with dyslipidemia and predisposing to atherosclerosis.

Because the promoter and 5’-untranslated region (5’-UTR) of the gene contain important regulatory regions for gene expression7 one might speculate that nucleotide changes in these regions of PPARD may result in altered mRNA and protein levels which, in turn, affect genes regulated by PPARD. Such functional polymorphisms in the human PPARD can be used as tools to elucidate the role of PPARD in lipid and lipoprotein metabolism in man. Accordingly, we hypothesized that variation at the PPARD locus might be associated with differences in plasma lipid and/or lipoprotein traits. In this study we screened the proximal promoter and the 5’-UTR for functional polymorphisms, one of which, a +294T/C polymorphism in exon 4, was associated with plasma LDL concentration in healthy men.

Methods

Human Subjects

Two clinical cohorts were investigated. First, a random sample of 543 healthy 50-year-old men living in the Northern part of the greater Stockholm area was examined8. The second cohort comprised 282 unselected healthy men, aged 41 to 60 (53.5±0.3) years, who were selected from the general population as control subjects in a case control-study of myocardial infarction. Specific exclusion criteria for both studies were plasma LDL–cholesterol concentration above 6.0 mmol/L, plasma triglycerides above the 95th percentile of the populations and blood glucose above 6.1 mmol/L. Only men of
North European descent were studied. These studies were approved by the Ethics Committee of the Karolinska Hospital. All subjects gave informed consent to participation.

Biochemical Measurements
Plasma lipid, lipoprotein, and apoB concentrations and whole blood glucose were measured as described.8 Plasma insulin was determined by an enzyme-linked immunosorbent assay (DAKO Diagnostics). Leptin was determined in EDTA-plasma samples with an enzyme-linked immunosorbent assay kit (Quantikine for human leptin) from R&D Systems. Intra- and interassay coefficients of variation for the leptin control plasma were 3% and 7%, respectively.

DNA Analysis
The proximal promoter, the 5'–UTR and exons 1 to 4 were amplified by polymerase chain reaction (PCR) in 20 healthy individuals and sequenced as described.1 The primers used were 2f and 7r for the proximal promoter and exon 1, 8f and 11r for exon 2, 12f and 14r for exon 3, and 15f and 19r for exon 4.1

The analyses of the −409C/T, +73C/T, and +255A/G PPAR and the L162V PPAR polymorphisms were performed by PCR amplifications. The forward primers were 2f, 91f (5'-GGACGAGC-GCGTGACCACGTT, 12f and PPARaf (5'-CCACTGTTGATTACCTCCTCGTTC, 15f and 19r for exon 3, and 15f and 19r for exon 4).1

Figures 1A and 1B. PCR products were digested with Bgl II (New England Biolabs) and the fragments were separated on a 2.5% agarose gel. ApoE genotype was determined as described.9

Cell Culture
The human monocytic cell line U937 was cultured in 7.5% FCS-RPMI 1640 medium with a supplement of penicillin (100 U/mL) and streptomycin (100 μg/mL) in standard culture flasks in a humidified 5% CO₂ system (Bio-Rad) and then immediately transferred to 7.5% FCS-RPMI 1640 medium (Life Technologies). At 24 hours post-transfection, the luciferase activity of all constructs was measured using a luciferase assay system (Promega).

DNA Constructs
A native promoter construct covering the region from −731 to +310 was cloned using a TA-cloning kit (Invitrogen). The T to C point mutation at +294 was introduced by a QuickChange Site-Directed Mutagenesis Kit (Stratagene). An EcoRI HindIII fragment was subsequently transferred to the pGL3-Basic Vector (Promega). Furthermore, a shorter promoter construct covering the proximal promoter from position −731 to +44 was also ligated into the pGL3-Basic Vector. Subsequent DNA sequencing confirmed that the sequences were in accordance with the genomic DNA sequence.1 Minimal and heterologous promoter constructs were created from two DNA fragments with flanking BamHI and BgII sites, covering the region from +283 to +310, which contains either the T or C site of the exon 4 polymorphism, and ligated into a Bgl II-restricted pGL3-Promoter Vector (Promega; Figure 1B).

Transient Transfection Assay
Plasmids were purified using an Endofree Plasmid Maxi kit (Qiagen). A total of 200 μL of cell suspension containing 20×10⁶ cells, 45 μg of the luciferase construct, and 1.2 μg of pRL-TK Vector (Promega) were transfected by electroporation using the GenePulser system (Bio-Rad) and then immediately transferred to 7.5% FCS-RPMI 1640 medium (Life Technologies). At 24 hours post-transfection, cells were plated at a density of 4.2×10⁶ cells/60-mm dish. Every dish received 3 μg of reporter construct, −731 to +310 bp with either the T or C allele, and 2 μg of the B-galactosidase expression plasmid p97b as an internal reference. Variable amounts of Sp-1 expression plasmid pPACSp-1 were compensated for by addition of empty pPAC expression plasmid. Transfected cells were incubated 24 hours before changing media. Cells were harvested 48 hours after transfection and lysed by 4 freeze-thaw cycles and centrifuged at 4°C. Luciferase and B-galactosidase assays were performed as described.11

Electrophoretic Mobility Shift Assay (EMSA)
The sequence of the double-stranded oligonucleotide used in the EMSA was 5'-GATATGATCCCTCTCCAGTGAGGA, 84f (5'-AGCCTGCGGAGCCGGCGG, 82f (5'-AAGCTAGAGCCCGTGGAC-A), and PPARaseq (5'-GTTGTTGATTCACACAAGTG), respectively, were used as sequencing primers.

Statistical Methods
Allele frequencies were estimated by gene counting. A chi-square test was used to compare the observed numbers of PPAR and PPARA genotypes with those expected for a population in Hardy–Weinberg equilibrium. One-way ANOVA and covariance analyses (with body mass index [BMI], insulin, or smoking as covariates) and two-way ANOVA were conducted to test whether genetic variation within the 5'-region of PPAR or the L162V PPAR polymorphism was associated with differences in plasma lipid and lipoprotein concentrations or anthropometric variables. The Sheffé multiple comparison test was used as post-hoc test. A 3×3 or 2×2 factorial ANOVA was computed to test for interaction between the PPAR and apoE or PPARA L162V genotypes, respectively. Differences in transcriptional activity between DNA constructs were evaluated by the nonparametric Mann–Whitney U test. All statistical analyses were performed using StatView (SAS Institute Inc., version 5.0.1).

Results
Identification of DNA Sequence Polymorphisms
To identify polymorphisms of functional importance in regulatory regions of PPAR, the proximal promoter and the
5'-UTR of the gene were examined. A genomic fragment comprising 731 bp of the proximal promoter and 44 bp of the 5'-UTR was cloned into the luciferase reporter vector and demonstrated to confer basal transcriptional activity in vitro compared with the vector alone (data not shown). A second postulated regulatory region for PPARD mRNA expression is the 5'-UTR. Accordingly, a region encompassing 731 bp of the proximal promoter and the first four exons comprising the 5'-UTR of PPARD were screened for polymorphisms in 20 unrelated subjects. Four polymorphisms were detected in this region: −409C/T in the promoter region, +73C/T in exon 1, +294T/C in exon 3, and +294T/C in exon 4 (Figure 1A). The T to C substitution at position +294 in exon 4 is located 86 bp upstream of the translation start site.

Association Studies
Genotyping was performed in 543 healthy 50-year-old men. All polymorphisms were found to be in Hardy–Weinberg equilibrium. The rare allele frequencies for the PPARD polymorphisms −409C/T, +73C/T, +294T/C, and +294T/C were 4.2%, 4.2%, 1.2%, and 15.6%, respectively. The PPARD promoter and exon 1 polymorphisms were found to be in almost complete allelic association (D’ = 0.96, P < 10^-5). The +294T/C polymorphism in exon 4 of PPARD showed a statistically significant association with LDL–cholesterol concentrations (Table 1). CC carriers had a significantly higher plasma LDL–cholesterol concentration than TT carriers (mean: CC 4.09, 0.23; TC 3.59, 0.07; TT 3.48, 0.04 mmol/L; P = 0.01). In addition, measurement of the plasma apoB concentration in a subset of 376 subjects showed that the C allele is associated with increased total apo B levels compared with the T allele, (mean: SE: CC 120.7, 7.1; TC 107.5, 2.4; TT 104.6, 1.3 mg/dL; P = 0.02). No additional associations were found between the +294T/C polymorphism and plasma concentrations of other lipids or lipoproteins, glucose, insulin, and leptin or with anthropometric variables, such as BMI (Table 1). Adjustments for BMI, insulin, and smoking status did not affect the relationships between the +294T/C polymorphism and any clinical or metabolic phenotype.

No genotype-specific differences in plasma concentrations of lipids or lipoproteins, glucose, insulin, and leptin or with anthropometric variables were observed for any of the −409C/T, +73C/T, or +294T/C polymorphisms (data not shown). Of note, the power of this study to detect significant differences in plasma LDL–cholesterol concentration was 80% for the +294T/C polymorphism but less than 25% for the other PPARD polymorphisms analyzed, as calculated from the mean LDL–cholesterol concentration of the respective genotypes and the standard deviation of the study group (0.8 mmol/L).

To validate the findings with the +294T/C polymorphism, 282 healthy men aged 41 to 60 years were genotyped for this polymorphism. In concert with our previous findings, individuals homozygous for the C allele had significantly higher LDL–cholesterol concentrations than carriers of the T allele (mean: SE: CC 4.31, 0.33; TC 3.40, 0.10; TT 3.57, 0.06 mmol/L; P = 0.02). The frequency of the rare C allele was 15.9%.

Gene–Gene Interactions
Potential interactive effects between the PPARD +294T/C and the apoE or PPARA L162V polymorphisms were stud-
ApoE genotypes were arranged in three groups (e2e2/e2e3, e3e3, and e3e4/e4e4) and PPARα L162V genotypes were studied using constructs covering the promoter and the 5’-UTR spanning nucleotides from −731 to +310 with the +294 T or C allele as well as a construct containing only the proximal promoter, position −731 to +44, which were transfected into human U937 monocytic cells. The C allele construct showed a 39% increase in transcriptional activity compared with the T allele construct (Figure 3A). Of note, the construct containing only the proximal promoter had still by far the highest promoter activity compared with the long T allele construct (Figure 3A).

To confirm the allele-specific differences, heterologous minimal promoter constructs containing oligonucleotides spanning the region from +281 to +310 with either the T or C allele were used (Figure 3B). The constructs were transfected into human U937 monocytic cells. The C allele construct now showed a small but still significant increase in basal transcriptional activity compared with the T allele (Figure 3B).

**Allele-Specific Regulation of PPARα**

Allele-specific differences in transcriptional activity were studied using constructs covering the promoter and the 5’-UTR spanning nucleotides from −731 to +310 with the +294 T or C allele as well as a construct containing only the proximal promoter, position −731 to +44, which were transfected into human U937 monocytic cells. The C allele construct showed a 39% increase in transcriptional activity compared with the T allele construct (Figure 3A). Of note, the construct containing only the proximal promoter had still by far the highest promoter activity compared with the long T allele construct (Figure 3A).

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**+294T/C Polymorphic Site Influences the Binding of Transcription Factor Sp-1**

To investigate whether the +294T/C polymorphism affects the interaction with nuclear proteins, EMSAs were performed. Probes of 25 bp of DNA, which contained either the +294T or +294C site, and nuclear extracts derived from human U937 monocytic cells were used. As shown in Figure 4, proteins present in the nuclear extract formed complexes with DNA probes (nucleotides from +281 to +305) and retarded their mobility. Proteins of the complex bound specifically to the rare +294C-probe and not to the common +294T-probe (Figure 4, lanes 2 to 3). Competition studies using a 150-fold excess of unlabeled probes showed that the protein complex with the fragment containing the C allele is sequence specific. As shown in Figure 4, the intensity of the band indicated with an arrow was unchanged when the T probe or an unspecific probe was added as competitor, whereas the band diminished almost completely when the C probe was used as competitor (Figure 4, lanes 5 to 7). No specific binding to the T allele was detected by competition experiments (Figure 4, lanes 13 to 15).

Database analysis of the sequence containing the polymorphic site revealed potential binding sites for the transcription factors Sp-1 and NF-κB on the rare C allele (Figure 1B; Transcription Element Search System; http://www.cbil.upenn.edu/tess). To confirm whether the GAACCAACCC sequence located between nucleotides +288 and +296 could bind Sp-1 and/or NF-κB, supershift analyses were performed with antibodies directed against Sp-1 and NF-κB, p50, p65, and c-Rel (Figure 4). The bands representing DNA–protein complexes were shifted with the anti–Sp-1 antibody (Figure 4, lane 8) whereas no supershift was noted with the anti–NF-κB antibodies (Figure 4, lane 9 to 11). Taken together, these results show that Sp-1 binds specifically to the rare C allele and not to the common T allele.

To further explore the impact of Sp-1 on the allele-specific effect on PPARα promoter activity, an Sp-1 expression vector was cotransfected with constructs covering the promoter and the 5’-UTR spanning from nucleotides −731 to...


+310 and containing either allelic variant. A Drosophila cell line that lacks Sp-1 activity was used to investigate the allele-specific effects of the ubiquitously expressed Sp-1. Sp-1 showed a dose–response effect on both the C and T allele (Figure 3C). With no Sp-1 present the C allele showed increased transcriptional activity compared with the T allele whereas presence of increasing concentrations of Sp-1 preferentially increased the transcriptional activity of the common T allele (Figure 3C). This implies that Sp-1 acts together with another, yet unidentified, factor in the allele-specific regulation of PPARD.

Discussion

Using a functional polymorphism we show for the first time that PPARD is implicated in human cholesterol metabolism. The proximal promoter and the 5'-UTR were screened for variations that alter the expression or function of PPARD to make an initial evaluation of the physiological significance of altered PPARD expression. Four polymorphisms were found in the 5'-region of PPARD: -409CT in the promoter: +73C/T in exon 1, +255A/G in exon 3, and +294T/C in exon 4. Only the +294T/C polymorphism showed a significant association with a metabolic trait in two independent and well-characterized cohorts consisting of a total of 825 healthy middle-aged Caucasian men. Homozygous carriers of the rare C allele had on average around 0.67 mmol/L higher plasma LDL–cholesterol concentration than homozygous carriers of the common T allele. Of note, plasma concentrations of LDL–cholesterol correlate directly with the incidence of coronary heart disease among healthy individuals. According to the Framingham risk algorithms, a 10% increase in total plasma cholesterol concentration results in a significantly increased risk of developing cardiovascular disease. This is in accordance with in vitro studies using a PPARD agonist, which demonstrated that PPARD promotes lipid accumulation in macrophages and that the agonist increases the expression of genes involved in lipid uptake and storage. Taken together, these data suggest that PPARD might play a role in the development of atherosclerosis.

It has recently been shown by in vitro studies that the three PPARs can either up or down regulate genes to different degrees depending on which PPAR is most effective in binding to the promoter region. This suggests that the balance between expression of both receptors and ligands might be of importance for the regulation of genes involved in lipid and glucose metabolism.

Thus far, the only published PPAR polymorphism implicated in cholesterol metabolism is the PPARA L162V. In type II diabetic patients, V162 allele carriers showed increased plasma total cholesterol, HDL–cholesterol, and apoAI concentrations. However, no associations with lipid parameters were detected in healthy subjects. Accordingly, we investigated whether the PPARA L162V polymorphism could interact with the PPARD +294T/C poly-

Figure 3. Differences in transcriptional activity between the T and C alleles of the +294 polymorphism. A, DNA constructs covering the region -731 to +310 of either T or C genotype or the proximal promoter, from position -731 to +44, were transfected into human monocytic U937 cells. The activity of the T allele is in every experiment set to 100% and the activities of the C allele and the proximal promoter are always compared with the activity of the T allele construct (n=6). B, DNA constructs covering the region +281 to +310 of either T or C genotype placed downstream of the SV40 promoter were transfected into U937 cells (n=3). C, DNA constructs covering the region -731 to +310 of either T or C allele were transfected into SL2 cells (n=3). All values are given as mean±SE. *P<0.05.

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morphism. Although no associations between the PPARA L162V polymorphism and plasma lipid or lipoprotein concentrations were detected, there was a marked interaction between the PPAR genotypes. Presence of the rare PPARA V162 allele significantly influenced the association between the PPARD +294C allele and the plasma concentration of LDL–cholesterol resulting in lower plasma LDL–cholesterol concentrations compared with subjects homozygous for the PPARD +294T allele. This is in contrast to the effect observed for the PPARD +294T/C polymorphism when it is examined in isolation where instead an increase in plasma LDL concentration was associated with the +294C PPARD allele. A similar interactive effect was found for plasma triglycerides, whereas an opposite effect was demonstrated on HDL–cholesterol resulting in higher HDL–cholesterol concentrations in carriers of both rare PPAR alleles.

The seemingly contradictory observations regarding the PPARA L162V might be explained by differences in metabolic status. It has been suggested that PPARA activity is not limiting in healthy subjects and therefore polymorphisms of PPARA are of minor importance for gene expression under normal metabolic conditions. Conversely, PPARA polymorphisms might have greater impact under circumstances with increased PPARA expression such as obesity and/or the insulin resistance syndrome.16 The role of PPARD has been investigated in obese rhesus monkeys that were treated with a potent selective PPARD agonist.4 Treatment with the agonist resulted in lowering of fasting plasma triglyceride levels, increased HDL–cholesterol concentrations and decreased fasting plasma insulin levels. This result is in agreement with the present study where interaction between PPARA and PPARD polymorphisms results in similar changes of lipid and lipoprotein concentrations. Of note, the agonist study by Oliver et al4 was conducted in obese monkeys whereas the present association study was performed in healthy men, and exclusion of obese individuals (BMI >30) from this cohort resulted in an even stronger association between the C allele and LDL–cholesterol (mean, SE: CC 4.11, 0.25; TC 3.62, 0.08; TT 3.47, 0.04 mmol/L; P = 0.004; n = 496).

The rationale for investigating the 5′-UTR was derived from findings regarding the regulation of the mouse Ppard. Transient transfection studies in 3T3-F442A preadipocytes and HEK293 kidney cells using luciferase constructs with the 5′-UTR showed a high level of transcriptional activity even without the promoter region, indicating that important promoter elements reside in the 5′-UTR. Of note, the human and murine PPARD structures are very similar.17

The present study showed that the +294T/C polymorphism influences the binding of the transcription factor Sp-1 and is associated with higher transcriptional activity for the rare C allele. The results obtained from transfections in Sp-1–deficient cells suggest that Sp-1 is not solely responsible for the higher promoter activity of the C allele. Because the transcriptional activity was differentially regulated by Sp-1 in SL2 cells, one might speculate that an as yet unidentified nuclear factor competes with Sp-1 for binding to the polymorphic site. A dose–response effect of overexpressed Sp-1 was shown for both alleles. This result reflects the presence of two additional potential Sp-1 consensus sites at positions −32 to −42 and −114 to −123.1

Considering that the human PPARD is located in the HLA complex on chromosome 6p, which is highly variable, it cannot formally be excluded that the +294T/C polymor-
pphism is in strong linkage disequilibrium, or even complete allelic association, with another as yet unidentified polymorphism located nearby that directly affects the plasma LDL—cholesterol concentration. Also, the power of this study to detect associations between the other three PPARD polymorphisms and the LDL—cholesterol concentration was low.

These restrictions notwithstanding, this is to the best of our knowledge the first study to indicate that PPARD plays a role in cholesterol metabolism in humans. It suggests that PPARD expression may be implicated in cardiovascular disease in humans.

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