Transcriptional Regulation of Apolipoprotein A5 Gene Expression by the Nuclear Receptor RORα.

Annelise Genoux[1], Hélène Dehondt[1], Audrey Helleboid-Chapman[1], Christian Duhem[1], Dean W. Hum[2], Geneviève Martin[2], Len Pennacchio[3], Bart Staels[1], Jamila Fruchart-Najib[1], and Jean-Charles Fruchart[1]

From the [1] Département d'Athérosclérose (A.G., H.D., A.H-C., C.D., B.S., J.F-N, J-C.F.), U.545 INSERM, Institut Pasteur de Lille and Faculté de Pharmacie de Lille, 1 rue Calmette, BP 245, 59019 Lille Cedex, France.

[2] Genfit SA (D.W.H., G.M.), Loos, France.

[3] Genome Sciences Department and Joint Genome Institute (L.P.), Lawrence Berkeley National Laboratory, Berkeley, USA.

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Corresponding author:

Jamila Fruchart-Najib

Phone: (33) 3 20 16 40 84

Fax: (33) 3 20 16 40 01

E-mail: jfruchart@pharma.univ-lille2.fr

Abbreviations*: APOA5*, the human apolipoprotein A5 gene; *apoa5*, the mouse apolipoprotein A5 gene; Apo A-V, the human protein; *APOC3*, the human apolipoprotein C3 gene; *apoc3*, the mouse apolipoprotein C3 gene; Apo C-III, the human protein; CHD, coronary heart disease

Objective - The newly identified apolipoprotein A5 (*APOA5*), selectively expressed in the liver, is a crucial determinant of plasma triglyceride levels. Since elevated plasma triglyceride concentrations constitute an independent risk factor for cardiovascular diseases, it is important to understand how the expression of this gene is regulated. In the present study, we identified the orphan nuclear receptor RORα as a regulator of human *APOA5* gene expression.

Methods and Results - Using electromobility shift assays, we first demonstrated that RORα1 and RORα4 proteins can bind specifically to a Direct Repeat 1 site present at the position –272/-260 in the *APOA5* gene promoter. In addition, using transient co-transfection experiments in HepG2 and HuH7 cells, we demonstrated that both RORα1 and RORα4 strongly increase *APOA5* promoter transcriptional activity, in a dose-dependent manner. Finally, adenoviral overexpression of hRORα in HepG2 cells led to enhanced *hAPOA5* mRNA accumulation. We showed that the homologous region in mouse *apoa5* promoter is not functional.

Conclusions - These findings identify RORα1 and RORα4 as transcriptional activators of human *APOA5* gene expression. These data suggest an additional important physiological role for RORα in the regulation of genes involved in lipid homeostasis and probably in the development of metabolic diseases such as atherosclerosis.

Condensed abstract: Apolipoprotein A5 has recently been identified as a crucial determinant of plasma triglyceride levels. Our results showed that RORα up-regulates human *APOA5* but has no effect on mouse *apoa5* promoter. These data suggest an additional important physiological role for RORα in the regulation of genes involved in plasma triglyceride homeostasis in human and probably in the development of atherosclerosis.

Key words: apolipoprotein A5, nuclear receptor RORα, triglyceride homeostasis, transcriptional regulation.

Cardiovascular diseases are major cause of death in the world.[1,2] Several epidemiological studies have established that, in addition to elevated low density lipoprotein and reduced high density lipoprotein levels, elevated blood triglyceride level is an independent risk factor for coronary heart disease (CHD).[3,4] Therefore, the identification of factors or genes capable of affecting triglyceride homeostasis is of major medical importance for the treatment of hypertriglyceridemia and associated-CHD.

The newly identified apolipoprotein A5 (*APOA5*) is located proximal to the well characterized APOA1/C3/A4 gene cluster on human 11q23. Predominantly expressed in the liver, the apolipoprotein A5 is an important determinant of plasma triglyceride levels both in humans and mice [5] and is associated with an early phase of liver regeneration.[6] To study the function of *APOA5* on triglyceride homeostasis, human *APOA5* transgenic and knock-out mice were generated.[5] Transgenic mice exhibited markedly decreased levels of plasma triglyceride that were about one third of those of control mice, whereas knock-out mice had about four fold increased plasma triglycerides compared to wild-type mice. In a previous study, we investigated the mechanism by which apo A-V affects lipid metabolism in transgenic mice over expressing *hAPOA5* gene.[7] We found that apo A-V does not affect VLDL triglyceride synthesis and secretion but accelerates VLDL catabolism by activating lipolysis and enhancing VLDL removal from the plasma.[7] Taken together, these findings demonstrate that *APOA5* regulates plasma triglyceride level only by increasing triglyceride catabolism *in vivo*. In addition, several DNA sequence polymorphisms in the gene have been found to be linked to increased plasma triglyceride levels,[8] and subsequently to an increased risk of myocardial infarction.[9] These data suggest *APOA5* as a strong modulator of triglyceride levels. Therefore, studying factors that regulate its expression is of significant clinical importance for the treatment of dyslipidemia.

It was previously described that *APOA5* gene expression is increased by PPARα (peroxisome proliferator-activated receptor) / RXR (retinoid X receptor) dimers that bind to a

Direct Repeat 1 (DR1) sequence located at the position –272/-260 in the *APOA5* gene promoter.[10,11] Recently, SREBP1c has been reported to down-regulate *APOA5* gene expression via a direct binding to the functional E-box present in human *APOA5* promoter.[12]

The ROR orphan receptors constitute a subfamily of orphan receptors encoded by three different genes, RORα, -β, and -γ.[13-15] RORs were initially reported to bind to response elements (RORE, retinoic acid receptor-related orphan receptor response element) consisting of a 6-bp A/T-rich sequence preceding the half-core PuGGTCA motif.[15-17] However, more complex RORE have also been described such as direct repeats of the PuGGTCA motifs preceded by a 6-bp A/T-rich sequence.[18] Due to alternative splicing and promoter usage, the RORα gene gives rise to 4 isoforms α1, α2, α3, and α4 (or RZRα),[14,15,19] which differ in their N-terminal domains and display distinct DNA recognition and transactivation properties.[15] In contrast to RORβ, only expressed in brain, retina and pineal gland,[20] both RORα and RORγ are widely expressed in peripheral tissues.[13,15,16,19] *Staggerer* mice (sg/sg) carry a natural deletion in the RORα gene that prevents the translation of its putative ligand-binding domain, thereby presumably disrupting the normal function of this transcription factor.[21] Interestingly, in addition to severe neurological disorders,[22] these mice display metabolic abnormalities.[23-25] These mice, maintained on an atherogenic diet, develop a severe hypo-alphalipoproteinemia and atherosclerosis.[25] Furthermore, RORα has already been involved in apolipoprotein A1 and C3 gene transcriptional regulation.[23,24] These data suggest an important role for RORα in lipoprotein metabolism and cardiovascular diseases.

In the present study, we demonstrated that both RORα1 and RORα4 isoforms are positive regulators of human *APOA5* gene transcription. Our data identify *APOA5* as a new target gene for RORα and suggest a role for RORα in plasma triglyceride homeostasis.

Methods

Cloning and Construction of Recombinant Plasmids

The human *APOA5* promoter fragment (-305/+62) was amplified by PCR using an *APOA5* genomic BAC clone [5,10] as template and cloned in the pGL3 luciferase vector. The followed forward oligonucleotide 5'-TCTGTTGGGGCCAGCCAG-3' and reverse oligonucleotide 5'-AATGCCCTCCCTTAGGACTGTGAC-3' primers were used for the PCR reaction. Site-directed mutagenesis of the construct (-305/+62) ($-270^{G\rightarrow C}$, $-263^{G\rightarrow A}$) was accomplished using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the recommendations of the manufacturer with the following oligonucleotides forward 5'-AGTGGGAAG**C**TTAAAG**A**TCATGGGGTT-3' and reverse 5'-AACCCCATGA**T**CTTTAA**G**CTTCCCACT-3'. The human wild-type *APOA5*-DR1 (-284/-243) (5'-AGGTCAGTGGGAAGGTTAAAGGTCATGGGGTTTGGGAGAAAC-3') oligonucleotide was cloned in four copies into the SV40-pGL3. The pCDNA3-hROR expression vectors have been previously described.[26] The constructs Ad-GFP and Ad-RORα1 as well as the corresponding adenoviral particles were prepared as described previously.[26] pAd-RORα4 and pAd-LacZ adenovirus were obtained by the Vira Power ™ Adenoviral Expression system (Invitrogen, Paislay, UK). Briefly, RORα4 and control LacZ cDNA were cloned in pAd DEST™-based expression vector (Bett *et al*, 1994). 293 A cells were transfected with pAd vectors to produce an adenoviral stock. This stock was amplified, purified on cesium chloride and titered by a plaque assay in 96 well plates to define the titer in pfu/ml.

Cell Culture, Viral Infection, and Transient Transfection Assays

Human hepatoma HepG2 and HuH7 cells were obtained from the European Collection of Animal Cell Culture (Porton Down, Salisbury, UK). Cell lines were maintained in standard

culture conditions (Dulbecco's modified Eagle's minimal essential medium, supplemented with 10% fetal calf serum, 5% non essential amino acids, and 5% sodium pyruvate (Invitrogen) at 37°C CO2, 95% air. Medium was changed every 2 days.

For infection experiments, HepG2 cells were seeded in 6-well plates at a density of $5x10^5$ cells/well and incubated at 37°C prior to viral infection. After seeding, viral particles were added at a multiplicity of infection of 100 and incubated for 3 h. HepG2 cells were infected with the Ad-RORα1 or Ad-RORα4 adenoviral expression vector, or with the same vector allowing expression of GFP (Ad-GFP, green fluorescent protein) or LacZ (Ad-LacZ, beta-galactosidase). Thereafter, cells were washed with PBS and incubated in culture medium for the indicated times. At the end of the experiment, cells were washed once with ice-cold PBS, lysed and scraped in 0.5 ml of ice-cold Trizol reagent.

For transfection experiments, HepG2 and HuH7 cells were seeded in 24-well plates at a density of 10^5 cells per well, and incubated at 37°C for 16h prior to transfection. Cells were transfected by the calcium phosphate co-precipitation procedure using 300 ng of reporter vector and different quantities (10, 30, 100, and 300 ng) of pCDNA3-hRORα1 and pCDNA3-hRORα4 expression vectors, or empty pCDNA3 plasmid vector as control. The total amount of DNA was kept constant by complementation with pCDNA3 empty vector. At the end of the experiment, the cells were washed with ice-cold PBS, and the luciferase (Luc) activity was measured using a luciferase assay system (Promega Corp., Madison, WI, USA).

Gel Retardation Assays

The pCDNA3-hRORα1, α2, α3, α4, and γ expression plasmids were in vitro transcribed using T7 polymerase and subsequently translated using rabbit reticulocyte lysate as recommended by the manufacturer (Promega). DNA-protein binding assays were conducted as described [27] using the following binding buffer: Hepes 10 mM, KCl 60 mM, glycerol 10%, MgCl2 5 mM, EDTA 0.5 mM, dithiothreitol 1 mM, PMSF 1 mM, poly(dIdC) 0.1 µg/µl,

herring sperm DNA 50 ng/µl containing 3 µl of programmed or unprogrammed reticulocyte lysate. Double-stranded oligonucleotides corresponding to the wild-type (wt) (forward 5'-GGGAAGGTTAAAGGTCATGGG-3' and reverse 5'-CCCATGACCTTTAACCTTCCC-3') or mutated (mt) (forward 5'-AGTGGGAAG**C**TTAAAG**A**TCATGGGGTT-3' and reverse 5'-AACCCCATGA**T**CTTTAA**G**CTTCCCACT-3') *hAPOA5*-DR1 response element present in the *hAPOA5* promoter and oligonucleotides corresponding to the wild-type m*apoa5* sequence (forward 5'-GGGGCGGTTGCTGGTCACAGGA-3' and reverse 5'-TCCTGTGACCAGCAACCGCCCC-3') were radio-labeled [γ-32P]ATP using T4 polynucleotide kinase and used as probes. For competition experiments, 25-, 100-, 200-, and 400-fold excess of cold wild-type or mutated probe was included 20 minutes before adding labeled probe. For supershift assays, 5 µL of 1X polyclonal RORα antibody (Santa Cruz Biotechnology, Le Perray en Yvelines, France) were added to the binding reaction. DNA binding complexes were resolved by 5% native polyacrylamide gel electrophoresis (PAGE) in 0.25X TBE. Gels were dried and exposed at –80°C to XOMAT-AR film (Eastman Kodak Co., Rochester, New-York, USA).

RNA Analysis Experiments

Total RNA extraction was performed with Trizol reagent (Invitrogen) as indicated by the manufacturer. RNA expression of *APOA5* and 36B4 genes was analyzed by real-time quantitative PCR using SYBR Green technology on a MX4000 apparatus (Stratagene Europe, Amsterdam, The Nederlands). PCR was performed with oligonucleotides forward 5'-ACGCACGCATCCAGCAGAAC-3' and reverse 5'-TCGGAGAGCATCTGGGGGTC-3' for human *APOA5* and forward 5'-CATGCTCAACATCTCCCCCTTCTCC-3' and reverse 5'-GGGAAGGTGTAATCCGTCTCCACAG-3' for 36B4. Quantification of *APOA5* mRNA levels was normalized using 36B4 as housekeeping gene.

Statistical Analysis

Results are presented as mean +/- SD (standard deviation). Statistical analysis was performed using t-tests and $p<0.05$ was considered statistically significant. Statistical differences from controls are indicated by asterisks (*, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$).

Results

RORα1 and RORα4 proteins specifically bind to a DR1 site in the human APOA5 gene promoter.

It was previously described that PPARα/RXR dimers bind to a DR1 site present at the position –272/-260 in the human *APOA5* gene promoter.[10,11] As this site presents structural homologies with a binding site for the orphan nuclear receptor hRORα, we evaluated whether hROR proteins translated in vitro could bind to this site by electromobility shift assay. First, a SDS-PAGE analysis was performed with the translated products in the presence of 35S methionine and demonstrated that all the ROR isoforms: α1,α2, α3, α4 and γ are synthesized in equivalent amounts (data not shown). As shown in Figure 1A, *in vitro* translated hRORα1 and RORα4 proteins bind on the DR1 site while RORα2, α3 and γ failed to bind this site. Further supershift experiments were performed with an antibody specific for RORα. As shown in Figure 1A, the binding was supershifted for RORα1 and eliminated for RORα4 with the antibody. These results confirm that the proteins which bind to the probe are indeed RORα isoforms. Using a labeled consensus probe that binds *in vitro* both monomers and dimers as reference, we determined that RORα1 and RORα4 bind as monomers to *hAPOA5* RORE-DR1 (data not shown). Interestingly, hRORα1 and RORα4 proteins, which are the only isoforms expressed in the liver,[28] can bind to the identified RORE (ROR Response Element), whereas hRORα2, hRORα3, and hRORγ proteins cannot bind to this response element (Figure 1A). To check the specificity of both hRORα1 and RORα4 proteins binding to the *hAPOA5* promoter RORE-DR1 site, electromobility shift assays using a radiolabeled mutated (-270$^{G\rightarrow C}$, -263$^{G\rightarrow A}$) DR1 site probe (Figure 1A) and competitions with unlabeled wild-type or mutated RORE-DR1 sites probes were performed (Figure 1B). As shown in Figure 1A, neither RORα1 nor RORα4 can bind to the mutated DR1 site. In addition,

unlabeled double stranded nucleotide corresponding to the wild-type RORE-DR1 site efficiently competed the binding of both RORα1 and RORα4 proteins to the RORE-DR1 site (Figure 1B). By contrast, unlabeled double stranded nucleotide corresponding to the mutated RORE-DR1 site failed to compete with binding of both RORα1 and RORα4 proteins to the RORE-DR1 site. These data indicate that the RORE-DR1 site present in the human *APOA5* gene promoter is indeed a binding site for RORα1 and RORα4 nuclear receptors.

Interestingly, when we aligned the human and other species promoter sequences, the *APOA5* RORE-DR1 site, as well as the *APOA5* PPRE site, was only conserved in the human, the chimpanzee and the baboon sequences (Figure 2A). Especially, 1 nucleotide difference was observed in the *APOA5* RORE-DR1 site in the mouse and rat sequences. Using an electromobility shift assay, we showed that *in vitro* translated ROR proteins don't bind to the mouse site (Figure 2B), suggesting that this site is nonfunctional in mice.

RORα1 and RORα4 enhance the activity of the human APOA5 gene promoter.

As RORα1 and RORα4 proteins specifically bind to a RORE-DR1 site present in the *hAPOA5* gene promoter, we investigated whether RORα1, α2, α3, α4 and γ can modulate *APOA5* gene promoter transcriptional activity. HepG2 and HuH7 human hepatocytes were transiently co-transfected with a Luciferase reporter vector driven by the human promoter fragment (-305/+62) (Figure 3A) and the different isoforms of ROR. The results in figure 3B (HepG2 cells) and 3C (HuH7 cells) showed, in contrast to RORα1 and RORα4, that none of RORα2, α3, γ had any effect on the *APOA5* promoter activity. These data are in agreement with the gel shift assays and show that only RORα1 and RORα4 are functional. We showed that the transcriptional activity of the *APOA5* reporter construct was strongly increased in a dose-dependent manner by the co-transfection of either RORα1 and RORα4 expression vectors in HepG2 cells (Figure 3D) and in HuH7 cells (Figure 3E). In order to demonstrate

that RORα1 and RORα4 increase the human *APOA5* promoter transcriptional activity by binding to the RORE-DR1 identified at the position –272/-260, HepG2 and HuH7 cells were transiently transfected with the Luciferase reporter vector driven by the mutated human *APOA5* promoter fragment -305/+62 ($-270^{G\rightarrow C}$, $-263^{G\rightarrow A}$). The response of the mutated construct to RORα1 or RORα4 was strongly decreased compared to the wild-type construct in HepG2 (Figure 4A) cells and in HuH7 cells (Figure 4B). These results suggest that the identified RORE-DR1 plays a key role in the regulation of the human *APOA5* transcriptional activity by RORα. However, since a 18% residual increase is still observed with the mutated construct, we cannot exclude that another mechanism independent of binding to this site may occur. To further demonstrate that the *APOA5* RORE-DR1 is functional, we cloned it in four tandem copies in front of a heterologous promoter and examined its response to RORα1 or RORα4 co-transfection in HepG2 and HuH7 cells. We found that this site could transmit RORα1 and RORα4 activator effect in a dose-dependent manner in HepG2 cells (Figure 4C) and in HuH7 cells (Figure 4D). Taken together, these results demonstrate that RORα1 and RORα4 increase *APOA5* promoter transcriptional activity mainly by a specific binding to the RORE-DR1 at position –272/-260.

Overexpression of hRORα1 and hRORα4 leads to increased hAPOA5 mRNA levels.

To confirm that hRORα1 and hRORα4 overexpression could also lead to increase accumulation of *hAPOA5* mRNA, HepG2 cells were infected with the Ad-RORα1 or the Ad-RORα4 adenoviral expression vector. As control, HepG2 cells were infected with the same vector allowing expression of GFP or LacZ. Total RNA was extracted and analyzed by real-time quantitative PCR using *hAPOA5* specific oligonucleotides and 36B4 as an internal control. As shown in Figure 5A, an increase of *hAPOA5* mRNA was observed in cells infected with the hRORα1 adenoviral expression vector compared with control cells infected

with Ad-GFP. In addition, the adenovirus-induced *hAPOA5* mRNA accumulation was shown to be time-dependent to reach a 6-fold increase after 48h incubation. A significant increase of *hAPOA5* mRNA was also observed in HepG2 cells infected with Ad-RORα4 compared with their control cells infected with Ad-LacZ, (Figure 5B). These results confirm that hRORα1 and hRORα4 play a key role in the physiological control of *APOA5* gene expression in human HepG2 cells.

Discussion

It is well established that hypertriglyceridemia constitutes an independent risk factor for cardiovascular diseases.[3,4] Since *APOA5* has been described as a crucial determinant of plasma triglyceride levels,[5] the identification of factors that regulate its expression is of major medical importance for the treatment of hypertriglyceridemia and associated-CHD. It has been reported that *hAPOA5* gene expression is up-regulated by the heterodimer PPARα/RXR by a direct binding to the Direct Repeat 1.[10,11] As this DR1 site presents structural homologies with the binding site for the orphan nuclear receptor ROR (Retinoic acid receptor-related Orphan Receptor), we investigated the role of RORα in the control of *hAPOA5* gene expression. Several studies attributed an important role to RORα in the regulation of lipid homeostasis by up-regulating some apolipoprotein gene expression as APOA1 and APOC3 at the transcriptional level [23,24] and recently caveolin-3 and CPT-I in skeletal muscle.[29] Moreover, RORα has been shown to play a role in lipid homeostasis in the vascular wall.[30] Besnard *et al.* demonstrated that RORα expression is significantly decreased in human atherosclerotic plaques.[30] In addition, sg/sg mice have an increased susceptibility to atherosclerosis [25] attributed to lower HDL plasma levels and to heightened inflammatory response.[26]

We demonstrated initially that both RORα1 and RORα4 can specifically bind to the identified RORE site –272/-263 present in the human *APOA5* gene promoter which consists of a perfect AGGTCA half-site core motif. The functional evaluation of this site, using transient co-transfection experiments with RORα and wild or mutated RORE in the context of the *hAPOA5* promoter, demonstrated that only RORα1 and RORα4 increase human *APOA5* promoter activity in HepG2 as well as HuH7 cells. In addition, *in vitro* experiments with Ad-RORα adenoviral expression vectors revealed that RORα1 and RORα4 overexpression lead to *APOA5* mRNA accumulation in HepG2 cells. Our data demonstrate that *APOA5* is a new

target gene for RORα1 and RORα4. These data could strengthen the role of RORα in lipid homeostasis.

The second objective of our study was to evaluate whether our results obtained in human could be extended to mouse. The *staggerer* mice (sg/sg), which carry a natural mutation in the RORα gene, exhibit an aberrant blood lipid profile associated with changes in plasma apolipoprotein levels. In particular, sg/sg mice display decreased apo C-III and triglyceride plasma levels.[25] As the triglyceride metabolism was affected in this mouse model, we addressed the question whether *apoa*5 gene is involved in this mechanism. First, we analyzed the alignment of the human and mouse promoter sequences which revealed that the mouse deviates from the human *APOA5* RORE-DR1 core motif identified at the position -272/-263 by 1 nucleotide. Moreover, EMSA and transient transfection experiments (data not shown) results suggested that this site is not functional in mice. These data indicated that RORα can not modulate *apoa5* gene expression in mice and that *APOA5* is a RORα target gene only in human.

In a recent study, it was demonstrated that apo A-V and apo C-III independently influence plasma triglyceride concentrations in an opposite manner.[31] It is well known that apo C-III, which is a component of VLDL, increases triglyceride levels via an inhibitory effect on lipoprotein lipase activity, and thus constitutes a risk factor for atherosclerosis.[32] The apo C-III has been reported to be up-regulated by RORα and these data rather argue against the potential protective effect of RORα in atherosclerosis. Interestingly, in our study, we demonstrated that RORα strongly increases *hAPOA5* gene expression in human hepatocytes, this new finding confers to RORα a beneficial role in the regulation of triglyceride metabolism that could counteract that of apo C-III. By regulating the gene expression of both apolipoproteins, RORα may play a determinant role, at least, in maintaining plasma triglyceride levels in humans. In mice, a different approach may be expected because apoc3 is

increased while *apoa5* seems to be unaffected. This hypothesis correlates with the lower circulating plasma triglyceride levels observed in sg/sg mice which could be due to the repression of apoc3 by the mutated RORα but not to the regulation of *apoa5* in this animal model.

In conclusion, we speculate that the up-regulation of *hAPOA5* expression might counteract the negative effect of the up-regulation of APOC3 by RORα in human and could avoid the elevation of plasma triglyceride levels. These new findings make of RORα a more attractive therapeutic target in the treatment of dyslipidemia and atherosclerosis.

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Figure legends

Figure 1. RORα1 and RORα4 proteins bind specifically to the DR1 located at the position (-272/-260) of the human *APOA5* gene promoter. Gel retardation assays were performed with the indicated end-labeled wild-type (wt) (-276/-256) **(A and B)** and mutant (mt) (-279/-253) **(B)** fragment of the human *APOA5* gene promoter in the presence of *in vitro* transcribed/translated human RORα1 and 4 **(A and B)**, human RORα2, α3 and RORγ **(A)**, or unprogrammed reticulocyte lysate as control (3 µl of each). Supershift assays were performed using 5 µL of a 1X polyclonal RORα specific antibody **(A)**. DNA-protein complexes were resolved by non-denaturing PAGE. Specific complexes, not observed with unprogrammed lysate, are indicated by arrows. Competition experiments **(B)** were performed in the presence (+) or absence (-) of unlabeled wild-type (Comp. wt, 25-, 100-, 200-, and 400-fold molar excess) or mutant (Comp. mt, 25-, 100-, 200-, and 400-fold molar excess) oligonucleotides.

Figure 2. RORα proteins do not bind to the mouse promoter homologous sequence. (A) The human and the other species promoter sequences homologous to the human RORE-DR1 were aligned. The sequence corresponding to the human PPRE-DR1 site AGGTTAAAGGTCA is in bold and the sequence corresponding to the human RORE half-core motif AGGTCA is underlined. **(B)** A gel retardation assay was performed with the wild-type (wt) fragment of the mouse *APOA5* gene promoter homologous to the human RORE-DR1 in the presence of *in vitro* transcribed/translated RORα1, RORα2, RORα3, RORα4 and RORγ or unprogrammed reticulocyte lysate as control (3 µl of each). DNA-protein complexes were resolved by non-denaturing PAGE. No specific complexe was observed.

Figure 3. RORα1 and RORα4 enhance the activity of the human APOA5 gene promoter in a dose-dependent manner. **(A)** The *APOA5* promoter (-305/+62) was used for the transient transfections. Exon 1 is depicted by a box with the start site of transcription indicated by +1. The RORE-DR1 site (-272/-260) is in bold and the two mutated nucleotides are underlined. HepG2 **(B)** and HuH7 **(C)** cells were transiently co-transfected with the wild-type human *APOA5* promoter fragment reporter plasmid (-305/+62) cloned in front of the Luciferase reporter gene (300 ng) and the pCDNA3-hRORα1, α2, α3, α4, γ expression plasmids (30 and 100 ng) or the empty pCDNA3 vector. HepG2 **(D)** and HuH7 **(E)** cells were transiently co-transfected with the wild-type human *APOA5* promoter fragment reporter plasmid (-305/+62) cloned in front of the Luciferase reporter gene (300 ng) and the pCDNA3-hRORα1 and pCDNA3-hRORα4 expression plasmids (10, 30, 100, and 300 ng) or the empty pCDNA3 vector as control (300 ng) for 24h.

Figure 4. The (-305/+62) region of the human *APOA5* gene promoter is activated by RORα1 and RORα4 mainly via the functional RORE-DR1 present at the position (-272/-260). HepG2 **(A)** and HuH7 **(B)** cells were transiently co-transfected with the mutated *APOA5* promoter fragment reporter plasmid -305/+62 ($-270^{G\rightarrow C}$, $-263^{G\rightarrow A}$) cloned in front of the Luciferase reporter gene (300 ng) and the expression plasmids pCDNA3-hRORα1, pCDNA3-hRORα4 (100 and 300 ng), or the empty pCDNA3 vector as control (300 ng) for 24h. HepG2 **(C)** and HuH7 **(D)** cells were transiently co-transfected with the reporter construct containing four tandem copies of the human *APOA5* RORE-DR1 inserted in front of an heterologous promoter cloned upstream of the luciferase reporter gene (300 ng) and the pCDNA3-hRORα1, pCDNA3-hRORα4 expression plasmids (100 and 300 ng) or the empty pCDNA3 vector as control (300 ng) for 24h.

Figure 5. Adenovirus-mediated overexpression of hRORα1 or hRORα4 leads to increased h*APOA5* mRNA accumulation in HepG2 cells. Human HepG2 cells seeded at a density of $5x10^5$ cells/dish were infected for 3h. As indicated, cells were infected with the Ad-RORα1 **(A)** or the Ad-RORα4 **(B)** adenoviral expression vector, or with the same vector allowing expression of GFP (Ad-GFP) or LacZ (Ad-LacZ), at a multiplicity of infection of 100. Then, cells were washed and incubated for the indicated time in standard culture medium. At the end of the experiment, cells were washed and lysed in Trizol reagent. Total mRNA was extracted and analyzed by quantitative RT-PCR on a Mx4000 apparatus (Stratagene). Results are expressed as % of control.