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Identification of the human ApoAV gene as a novel RORα target gene

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Abstract

Retinoic acid receptor-related orphan receptor-α (RORα) (NR1F1) is an orphan nuclear receptor with a potential role in metabolism. Previous studies have shown that RORα regulates transcription of the murine Apolipoprotein AI gene and human Apolipoprotein CIII genes. In the present study, we present evidence that RORα also induces transcription of the human Apolipoprotein AV gene, a recently identified apolipoprotein associated with triglyceride levels. Adenovirus-mediated overexpression of RORα increased the endogenous expression of ApoAV in HepG2 cells and RORα also enhanced the activity of an ApoAV promoter construct in transiently transfected HepG2 cells. Deletion and mutation studies identified three AGGTCA motifs in the ApoAV promoter that mediate RORα transactivation, one of which overlaps with a previously identified binding site for PPARα. Together, these results suggest a novel mechanism whereby RORα modulates lipid metabolism and implies RORα as a potential target for the treatment of dyslipidemia and atherosclerosis.

Keywords: ApoAV; RORα; Transcriptional regulation; Nuclear receptor; Lipid metabolism

Retinoic acid receptor-related orphan receptor-α (RORα) is a widely expressed receptor that belongs to a subgroup within the family of nuclear receptors consisting of the three genes RORα, β, and γ [1]. The human RORα gene encodes at least four splicing isoforms, RORα1, α2, α3, and α4, that differ only in their N-terminal domains [2–4]. RORα binds as a monomer or homodimer to regulatory sequences (ROREs) composed of one core motif AGGTCA or two direct AGGTCA-repeats spaced by two nucleotides preceded by a six nucleotide long AT-rich sequence [2,4–9]. Several target genes of RORα containing such regulatory sequences have been identified including Rev-erbα, Ik-Bα, N-myc, laminin B1, and γF-crystallin [5,10–14].

Two putative ligands have been proposed for RORα, the natural hormone melatonin and synthetic thiazolidine diones [15–17]. Both types of ligands have been claimed to increase the transcriptional activity of RORα, but the data are highly controversial and RORα is therefore still considered as an orphan nuclear receptor. Kallen et al. [18] have recently solved the 3D structure and co-crystallized cholesterol in the ligand-binding pocket of RORα. Moreover, the transcriptional activity of RORα could be modulated by changes in intracellular cholesterol levels or by mutation of RORα amino acid residues involved in cholesterol binding [18]. Cholesterol sulfate was identified as the most active form among the cholesterol derivatives capable of activating RORα [18,19]. Whether RORα has a role in the regulation of cholesterol homeostasis remains unclear.

Although RORα has been suggested to be involved in a range of functions such as lipid metabolism, inflammation control, bone metabolism, and myocyte differentiation [20–24], the physiological role of RORα is not fully

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understood. The staggerer mice, which contain a natural deletion within the RORα gene leading to a truncated receptor, exhibit abnormal pro-inflammatory cytokine production, cerebral atrophy, and altered apolipoprotein profiles [21,22,25–29]. They also display an increased susceptibility to develop atherosclerosis when fed a high fat diet [30]. Despite the severe brain phenotype of the staggerer mice and hence difficulty in interpreting metabolic data from this model, the increased susceptibility to develop atherosclerosis suggests that RORα might have a protective role against development of dyslipidemia and ensuing artherosclerosis.

Plasma levels of ApoAI, which is the major component of HDL, as well as the mRNA levels of ApoAI in the intestine were reduced in the staggerer mice [22]. Surprisingly, triglyceride levels were also reduced, which was suggested to be caused by the observed decrease in ApoCIII levels [21]. The mRNA levels of ApoCIII were also reduced in both liver and intestine [21]. In vitro studies showed that the rat ApoAI and human ApoCIII genes are direct targets for RORα [21,22]. ROREs were found in their promoters to which RORα was shown to bind and RORα could activate transcription of reporter plasmids containing the rat ApoAI or human ApoCIII promoter [21,22]. The RORE found in the rat ApoAI promoter is not completely conserved in mouse and human, and the human promoter was shown not to be activated by RORα, indicating that RORα regulation of ApoAI transcription might be species dependent [22,31].

In the genome, ApoAI and ApoCIII are localized together with ApoAIV in the ApoAI/AIV/CIII gene cluster and mutations within this gene cluster have been shown to affect plasma levels of both cholesterol and triglycerides [32]. Recently, a new apolipoprotein, ApoAV, was identified by two independent groups and was found to be localized in close proximity to the ApoAI/CIII/IV cluster [33,34]. ApoAV is mainly expressed in liver and several studies indicate that its expression is inversely correlated with triglyceride levels [35,36]. ApoAV knockout mice have a fourfold increase in plasma triglyceride levels, whereas transgenic mice overexpressing human ApoAV or mice infected with an adenoviral vector expressing mouse-ApoAV showed a 70% decrease in triglyceride levels [35,36]. In addition, polymorphisms in the human ApoAV gene have been associated with elevated triglyceride levels as well as with low HDL levels and particle size of LDL [33,37–49].

ApoAV expression was recently shown to be regulated by peroxisome proliferator-activated receptor PPARα, a nuclear receptor that is activated by fatty acids and their derivatives [50,51]. PPARα binds as a heterodimer with RXR to peroxisome proliferator response elements (PPREs) within promoter regions of its target genes, many of which are involved in lipid and energy metabolism [52]. A PPRE consisting of a direct repeat of two AGGTCA motifs spaced by one nucleotide (DR-1) was identified in the ApoAV promoter [50,51]. A binding site for farnesoid X-activated receptor (FXR), a nuclear receptor involved in bile acid homeostasis, was also found, but endogenous ApoAV levels were not induced by an FXR ligand [50].

The previous finding that RORα could regulate transcription of ApoAI and ApoCIII, which are located in the same gene cluster as the recently identified ApoAV, together with the fact that other nuclear receptors were shown to regulate ApoAV transcription, led us to investigate if RORα could also induce ApoAV transcription.

Material and methods

Plasmids. pcDNA3.1-RORα1 was constructed by inserting a fragment containing the coding sequence of human RORα1 preceded by an ACC (as part of the Kozak sequence) into the *Hin*dIII–*Bam*HI site of pcDNA3.1 hygro+ (Invitrogen). hRXRα was cloned by PCR from spleen cDNA into pCR2.1 using the primers 5′-GGCAT GAGTTAGTCGCAGACA-3′ (fw) and 5′-CTGAGAAGAACAG CTGGCGT-3′ (rev), and then subcloned into pcDNA3.1 using the *Hin*dIII and *Xba*I sites to create pcDNA3.1-RXRα.

pcDNA3-PPARα was constructed by amplifying the PPARα cDNA from a human liver cDNA library (Stratagene) using gene specific primers and cloned into the *Kpn*I and *Bam*HI restriction sites in pcDNA3 (Invitrogen).

pRL-TK, expressing *Renilla* luciferase and used as an internal control in transfections of HepG2 cells, was obtained from Promega. The ApoAV promoter was cloned by PCR from placental human genomic DNA (Clontech) using primers ApoAV-356 (5′-AAGTGA GCTCTGGGTAGTTGTGTAAGAGAG-3′) and ApoAV +62 (5′-TTAACTCGAGAATGCCCTCCCTTAGGACTGT-3′). This construct that contains the sequence from −356 to +62 with respect to the transcriptional start site was then cloned into pGL3basic (Promega) using the *Sac*I and *Xho*I sites.

Mutation of the ApoAV promoter yielding constructs ApoAV R1mut, ApoAV R2mut, ApoAV R3 mut, and ApoAV R4 mut was performed with the Quick Change mutagenesis kit (Stratagene) using the sense primers: 5′-GTTGGTGGGCCAGCCAGC**GAACTC**GTG GGAAGGTTAAAGGTC-3′, 5′-GGTCAGTGGGAAGGTTAA**GA ACTC**TGG GGTTTGGGAGAAACTG-3′, 5′-GAGTGCTGGGAG GCAGCTG**GAACTC**ACTTCTTTTGAACTTCCACG-3′, and 5′-G CTGGGGCAGAGGGATGG**GAACTC**CAGTCCTAAGGGAGGG CA-3′, respectively. The respective antisense primers were used together with sense primers in the mutagenesis reaction. The construct ApoAV R1 + R2 mut was generated by a second round of mutations on the ApoAV R2 mut construct using the ApoAV R1 mut primers.

Deletion constructs −278/+62, −259/+62, and −97/+62 of the ApoAV promoter were produced by PCR of the −356/+62 construct using primer ApoAV+62 together with primers 5′-TAATGAGCTCG TGGGAAGGTTAAAGGTCATG-3′, 5′-TAATGAGCTCTGGGG TTTGGGAGAAACTGG-3′, and 5′-TAATGAGCTCACTTCTTTT GAACTTCCACGT-3′, respectively.

Recombinant adenovirus vectors were generated by inserting the cDNAs encoding RORα or ZsGreen (Clontech) into the pENTR1A vector (Invitrogen) together with the CMV promoter and the bGH polyA sequence from pcDNA3.1. The mock control contained only the CMV promoter and the bGH polyA sequence, without any cDNA insert. The resulting plasmids were recombined in vitro with the pAd/PL-DEST vector containing an E1 and E3 deleted adenovirus genome (ViraPower Adenoviral Expression System from Invitrogen). All constructs were confirmed by sequencing.

Cell culture, transient transfections, and luciferase assays. 293A cells (Invitrogen), used for adenovirus production, were grown in DMEM with high glucose (Gibco) supplemented with 10% fetal calf serum.

HepG2 cells were grown in MEM (Eagle) with glutamax-1 (Gibco) supplemented with 10% fetal calf serum, 1% sodium pyruvate (Gibco), and 1% non-essential amino acids (Gibco) at 37 °C in a humidified atmosphere with 5% CO_2. 16–24 h before transfection, cells were seeded in a 12-well plate at 1.2×10^5 cells per well. Cells were then transfected with 1 μg luciferase reporter plasmid containing wild-type, mutated or deleted ApoAV promoter together with 500 ng pcDNA3.1-RORα1 and 50 ng pRL-TK using Fugene (Roche). When performing RORα1 dose–response curves, 100–500 ng pcDNA3-RORα1 was added. The total amount of transfected DNA was held constant by adding the empty vector pcDNA3.1. After 5.5 h the medium was changed and the cells were incubated for a further 48 h. Cells were then washed with PBS and lysed by adding 250 μl passive lysis buffer (Promega). After 15 min incubation on a shaking platform, 20 μl of cell extracts was assayed for luciferase activity using a Dual-luciferase Reporter assay system (Promega).

In vitro translation and EMSA. In vitro translated RORα1 was produced from pcDNA3.1-RORα1 using the TNT Coupled Reticulocyte Lysate system from Promega.

Labelling of oligonucleotides for electrophoretic mobility shift assay (EMSA) was achieved by mixing 1 pmol oligo, 3U T4 Polynucleotide Kinase (USB), 1.5 μl T4 PNK 10× reaction buffer (USB), and 2 μl [γ-^{32}P]ATP (Amersham-Pharmacia Biotech (6000 Ci/mmol)) in a total volume of 15 μl. The labelling reaction was incubated for 30 min at 37 °C. To obtain double stranded DNA, 1 pmol unlabelled antisense oligonucleotide was added and the mix was heated to 95 °C for 5 min. The temperature was then slowly decreased to below 30 °C. To remove free nucleotides, Microspin G50 columns from Amersham Biosciences were used according to the supplied protocol.

Binding of the in vitro translated RORα1 to the ^{32}P-labelled oligo was carried out in a total volume of 15 μl containing 10 mM Tris–HCl, pH 8, 40 mM KCl, 6% glycerol, 1 mM DTT, 0.05% Triton X-100, 1 μg Poly(dI–dC) (Amersham-Pharmacia Biotech), 2 μl in vitro translated RORα1, and 20,000 cpm (around 3 fmol) labelled oligo. The reaction was incubated for 30 min at room temperature. When binding competition was performed, labelled oligo was not added until the unlabelled competitor (1000×–25,000×) had been incubated together with the other reagents for 10 min. The total reaction mixture was then incubated for another 20 min. The binding reaction was loaded together with loading buffer (High density TBE sample buffer 5×, Invitrogen) onto a 6% polyacrylamide gel (DNA retardation gel from Invitrogen) and run at 90 V for about 90 min in 0.25× TBE. Phosphor screens were exposed to dried gels and scanned in a Phosphorimager (Molecular Dynamics).

The sense oligos used in the EMSAs were: RORE; 5′-TCGA CTCGTATAACTAGGTCAAGCGCTG-3′, R1; 5′-GGGCCAGCC AGCAGGTCAGTGGGAAGGT-3′, R2; 5′-TGGGAAGGTTAAA GGTCATGGGGTTTGG-3′, R3; 5′-GGGAGGCAGCTGAGGTCA ACTTCTTTTG-3′ R4; and 5′-CAGAGGGATGGGGGTCACAGT CCTAAGG-3′, R1mut; 5-′ GGGCCAGCCAGC**GAACTC**GTGGG AAGGT-3′, R2mut; 5′-TGGGAAGGTTAA**GAACTC**TGGGGTTT GG-3′, R3mut; 5′-GGGAGGCAGCTG**GAACTC**ACTTCTTTTG-3′; and R4mut; 5′-CAGAGGGATGG**GAACTC**CAGTCCTAAGG-3′.

Adenovirus production and viral transduction of HepG2 cells. Recombinant adenovirus vectors were transfected into the 293A cell line (Invitrogen), according to the ViraPower Adenoviral Expression System protocol, to produce replication-deficient adenovirus. The virus was amplified to generate a high-titer virus stock. Viral titers were determined by Adeno-X Rapid titer kit (BD Biosciences) and defined as infectious units (iu)/ml.

HepG2 cells were transduced at a multiplicity of infection (MOI) of 500 iu/cell. After 3.5 h virus containing medium was withdrawn and fresh medium was added. Cells were harvested 48 h later and total RNA was prepared using Trizol reagent (Invitrogen). After DNase treatment with DNA*free* (Ambion), cDNA was synthesized using SuperScript First Strand Synthesis System for RT-PCR (Invitrogen).

Real-time PCR analysis. Real-time PCRs contained cDNA corresponding to 5 ng total RNA, 0.4 μmol/L each of the primers, 0.1 μmol/L of the probe, and TaqMan Universal PCR Master Mix (PE Applied Biosystems), and were performed with an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems).

Primers for ApoAI were: 5′-CAGAGACTATGTGTCCCAG TTTGAA-3′ (forward) and 5′-GTCCCAGTTGTCAAGGAGCTTT-3′ (reverse). The probe sequence was: 5′-CTCCGCCTTGGGAAAAC AGCTAAACC-3′. Primers for ApoCIII were: 5′-CACCAAGACC GCCAAGGAT-3′ (forward) and 5′-TAACGGTGCTCCAGTAGT CTTTCA-3′ (reverse). The probe sequence was: 5′-TCGGTCACCCA GCCCCTGGC-3′. Primers for ApoAIV were: 5′-GGAGGCCGTGG AACATCTC-3′ (forward) and 5′-TTCTCCAAGTTTGTCCTGGAA GA-3′ (reverse). The probe sequence was: 5′-AGAAATCTGAACTC ACCCAGCAACTCAATGC-3′. Primers for ApoAV were: 5′-GAGC AGATCCATCAGCAGAAGA-3′ (forward) and 5′-CCTCAGCTTT TCCAGGAACTTGT-3′ (reverse). The probe sequence was: 5′-CGAGCCCGCGACCCTGAAAGA-3′. Primers for Rev-erbα were: 5′-GCAAGAGCACCAGCAACATC-3′ (forward) and 5′-GGCAA CGTCCCCACACA-3′ (reverse). The probe sequence was: 5′-CACA GTAACACCATGCCATTCAGCTTGG-3′. Primers for the internal control h36B4 (human acidic ribosomal phosphoprotein) were: 5′-CCATTCTATCATCAACGGGTACAA-3′ (forward) and 5′-AGCA AGTGGGAAGGTGTAATCC-3′ (reverse). The probe sequence was 5′-TCTCCACAGACAAGGCCAGGACTCGT-3′.

The relative expression levels were calculated according to the formula $2^{-\Delta C_t}$, where ΔC_t is the difference in C_t values between the target and the 36B4 internal control.

Results

To investigate if RORα could regulate transcription of ApoAV, we started by searching the promoter region (−1936 to +62) for potential binding sites for RORα (AGGTCA or GGGTCA). Three AGGTCA motifs (R1–R3) and one GGGTCA motif (R4) were found, all located within the region −284/+62 (Fig. 1). At least one motif (R2) was preceded by an AT-rich sequence and therefore most closely resembled an RORE. R2 is also part of the DR-1 sequence to which PPARα has

Fig. 1. Sequence of the ApoAV promoter region (−356/+62). R1–R4 represents four potential binding sites for RORα1 (A/GGGTCA). +1 defines the predicted transcriptional start site.

been shown to bind. The sequence corresponding to −356/+62 of the ApoAV promoter was then cloned into the luciferase reporter plasmid pGL3basic. Co-transfection of increasing amounts of a RORα mammalian expression construct (pcDNA3.1-RORα1) and the ApoAV-promoter driven reporter construct into HepG2 cells showed that RORα activated transcription through the promoter in a dose–dependent manner (Fig. 2). Addition of RORα to the promoterless pGL3basic vector had no effect, showing that the RORα-induced reporter activity was specifically mediated through interaction with the ApoAV-promoter.

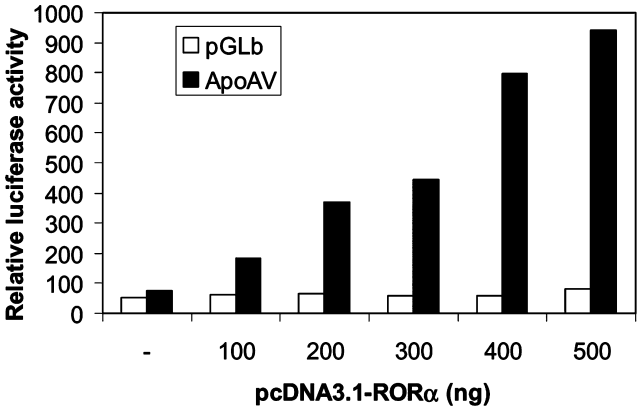

Fig. 2. RORα-dependent regulation of the ApoAV promoter in HepG2 cells. Increasing amounts of pcDNA3.1-RORα1 were co-transfected with empty pGL3basic vector (pGLb) or pGL3basic containing the ApoAV-promoter (−356/+62) into HepG2 cells. The luciferase-reporter activity was normalized against *Renilla* luciferase that was used as a transfection efficiency control. One of two independent experiments with similar results is shown.

To identify which promoter elements were important for the RORα-dependent transcription, deletion studies were performed. The three AGGTCA motifs were sequentially removed from the ApoAV promoter luciferase reporter constructs (Fig. 3). Deletion of the R1 element reduced the activity to approximately 30% of the activity obtained by RORα on the wild-type −356/+62 promoter–reporter construct. Deletion of both the R1 and R2 elements did not result in a further reduction in activity and deletion of all three elements (R1, R2, and R3) reduced activity to almost background levels (20%) (Fig. 3). Therefore, the R1 element appears to be most important for the RORα-dependent activation of the ApoAV promoter, since its removal decreases activity substantially and further deletions do not result in a significant, additional loss of activity. However, to investigate the role of the other elements, including R2 which most closely resembles an RORE, mutations of all four elements were performed in the context of the promoter. The six nucleotides in the core motif AGGTCA were changed to GAACTC for each element (Fig. 4A). The R1 and R2 mutations were also tested in combination. Mutation of either of the R1, R2 or R3 elements reduced the activity to between 40% and 60% of the wild-type activity, whereas mutating R4 did not reduce activity. Mutation of the R1 and R2 elements in combination reduced the activity to 25% of the wild-type activity, which is close to background level (Fig. 4A).

The R2 element is part of the DR-1 site to which PPARα has been shown to bind [50,51]. Therefore, the effect of mutating R2 and the closely spaced R1 on PPARα activation of the ApoAV reporter construct was tested. In agreement with previous studies, we

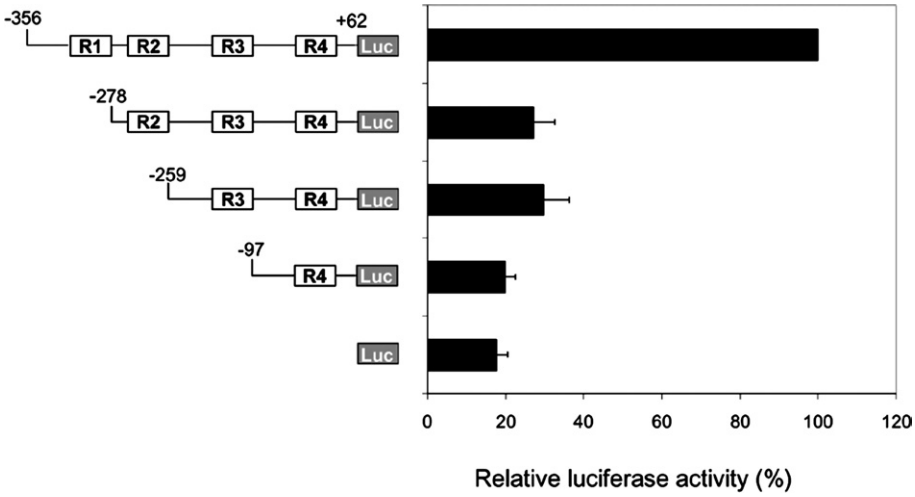

Fig. 3. Deletion of potential ROREs in the ApoAV promoter. The three AGGTCA motifs (R1–R3) in the ApoAV promoter were sequentially deleted, yielding promoter constructs containing the sequences −278/+62, −259/+62, and −97/+62. These promoter constructs were co-transfected with pcDNA3.1-RORα into HepG2 cells. The relative activity shown is the ratio between the fold induction in activity obtained by adding RORα to a deleted promoter construct and the fold induction in activity obtained by adding RORα to the wild-type promoter construct. Each data point is an average of at least five independent experiments with triplicate samples.

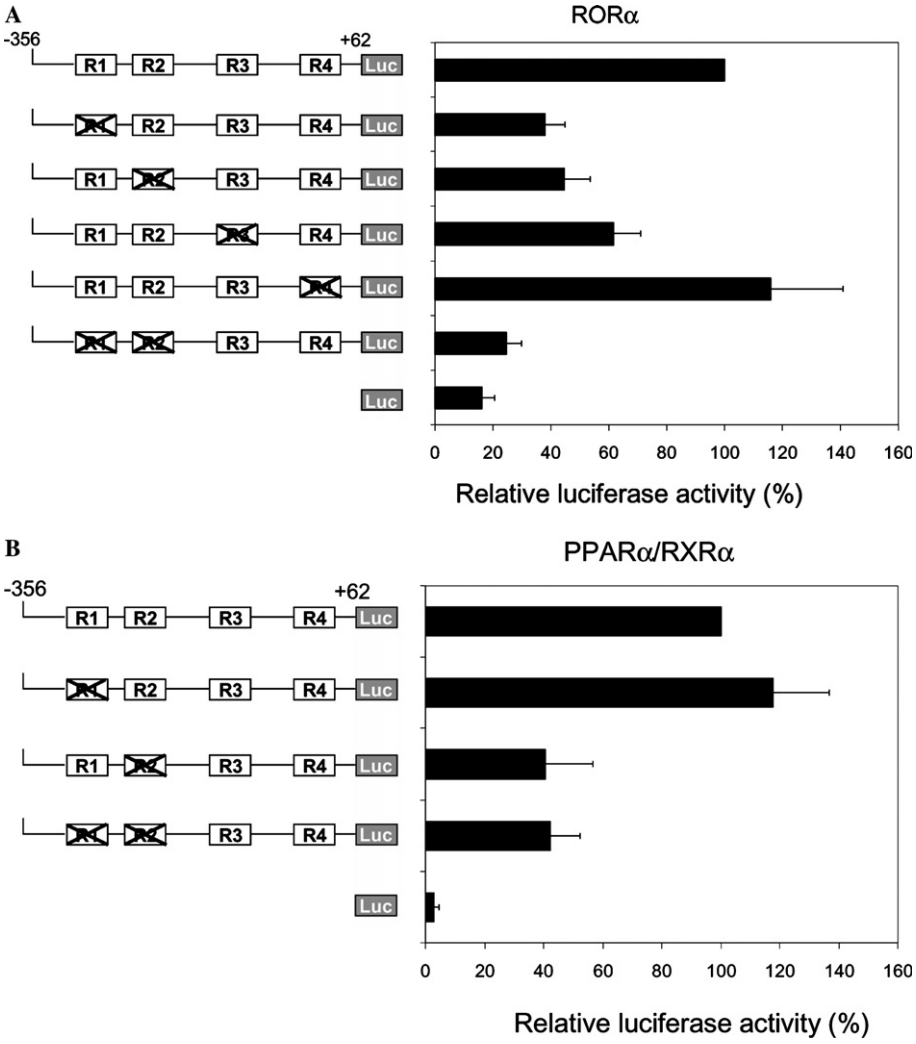

Fig. 4. Mutation of potential ROREs in the ApoAV promoter. The R1, R2, R3 (AGGTCA), and R4 (GGGTCA) motifs in the ApoAV promoter were mutated to GAACTC. (A) The mutated R1, R2, R3, and R4 constructs were co-transfected with pcDNA3.1-RORα into HepG2 cells. (B) The R1 and R2 constructs were co-transfected with pcDNA3-PPARα and pcDNA3.1-RXRα into HepG2 cells. The relative activity shown is the ratio between the fold induction in activity obtained by adding RORα or PPARα/RXRα to each mutated promoter construct and the fold induction in activity obtained by adding RORα or PPARα/RXRα to the wild-type promoter. Each data point is an average of at least five independent experiments with triplicate samples.

demonstrated that unliganded PPARα/RXRα activates the ApoAV promoter and that mutation of the R2 element greatly reduces the activity (Fig. 4B). As expected, mutation of the R1 element did not affect the activity.

To investigate if RORα bound to any of the elements, EMSA competition studies were carried out using increasing amounts of unlabelled R1, R2, R3 or R4 containing oligonucleotides to compete for binding with a ^{32}P-labelled synthetic RORE (Fig. 5). The wild-type R1, R2, and R3 elements competed for RORE binding, whereas the R4 element did not. The R1 and R2 competed for binding with somewhat higher affinity than the R3 element, but still with substantially lower affinity than the synthetic RORE. Elements containing mutations of the AGGTCA-motif did not compete, indicating that the binding was specific (Fig. 5). RORα thus binds to the R1, R2, and R3 elements in vitro, but not to the R4 element, which supports the data from the mutational analysis, where the intact R1–R3 elements, but not the R4 element, were necessary for full activation of the ApoAV promoter.

Having shown that RORα could activate an ApoAV promoter driven reporter gene, we wanted to investigate if RORα overexpression could induce endogenous ApoAV gene transcription. RORα was therefore overexpressed in HepG2 cells, using an adenoviral gene delivery system (Fig. 6). The expression of RORα1 was confirmed using Western blot (data not shown). The mRNA level of ApoAV was increased more than 3-fold in the cells transduced with RORα recombinant adenovirus compared to cells transduced with adenovirus without an insert (mock) or with the

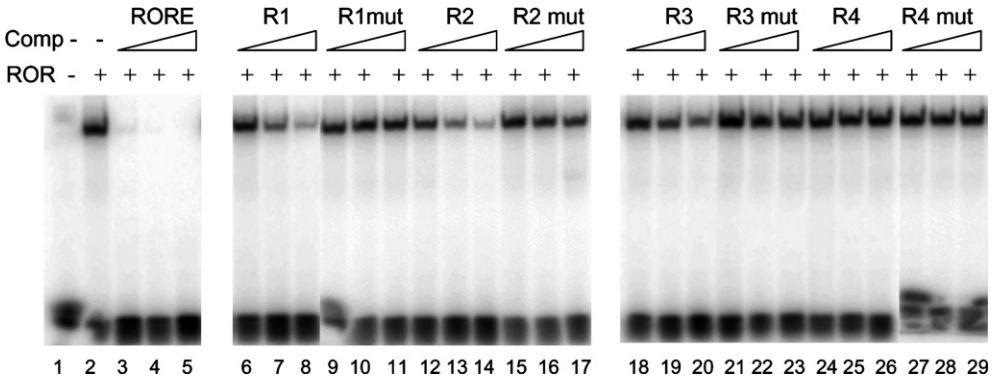

Fig. 5. RORα binds to the R1, R2, and R3 AGGTCA motifs in the ApoAV promoter. EMSA was performed using in vitro translated RORα1 (ROR+) or unprogrammed lysate (ROR−) and a ^{32}P-labelled oligonucleotide containing a consensus RORE. For competition, unlabelled oligonucleotides containing the consensus RORE (lanes 3–5), R1 (lanes 6–8), R1 mut (9–11) R2 (lanes 12–14), R2 mut (lanes 15–17), R3 (lanes 18–20), R3 mut (lanes 21–23), R4 (lanes 24–26) or R4 mut (lanes 27–29) were added at 1000, 10,000, and 25,000 molar excess. One of three experiments with similar results is shown.

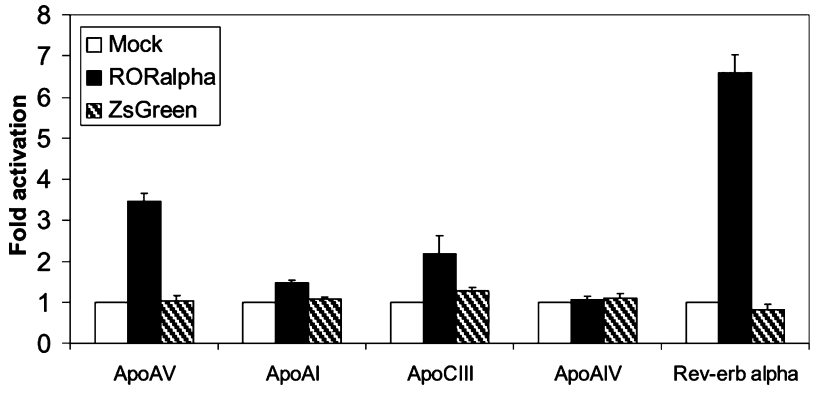

Fig. 6. RORα overexpression in HepG2 cells induces endogenous ApoAV expression. HepG2 cells were transduced with recombinant adenovirus expressing RORα or the fluorescent protein ZsGreen. Total RNA was prepared 48 h after transduction and the expression levels of ApoAV, ApoAI, ApoCIII, ApoIV, and Rev-erbα were measured by real-time PCR. Results are shown as fold activation relative to the expression level obtained from the mock control. Each data point is an average value of at least four independent experiments.

fluorescent protein ZsGreen. In addition, ApoCIII levels were increased about 2-fold in agreement with previous promoter studies and the observed decreased ApoCIII mRNA levels in staggerer mice. ApoAI levels were slightly increased approximately 1.4-fold. Whether RORα regulates ApoAI in humans needs to be further investigated, since RORα did not induce transcription from the human ApoAI promoter [31]. No effect on ApoAIV expression was seen. As previously reported, overexpression of RORα1 in HepG2 cells markedly induced expression of Rev-erbα to about 7-fold (Fig. 6) [5]. These results, together with the results from the promoter analysis, support that ApoAV is a target gene for RORα.

Discussion

RORα has previously been shown to regulate transcription of the rat ApoAI and human ApoCIII genes and we have in this study identified the human ApoAV gene as a new target gene for RORα. RORα activated an ApoAV promoter driven luciferase construct as well as induced endogenous levels of ApoAV mRNA when overexpressed in HepG2 cells. RORα was also shown to bind to three AGGTCA motifs in the ApoAV promoter located between positions −284 and −98 with respect to the transcriptional start site. The binding affinity of RORα in EMSAs for either element was relatively low compared to a consensus RORE. However, it is possible that other transcription factors/coregulators present in vivo increase the binding affinity and/or that the presence of three closely spaced elements compensates for low affinity. Only one element (R2) resembles a classical RORE where the AGGTCA motif is preceded by an AT-rich sequence. However, the affinity of RORα for the R2 element is no higher than for the R1 element, which is not preceded by such a sequence. Possibly, the exact composition of the AT-sequence is of importance [2]. Our results suggest that RORα can bind to AGGTCA motifs not preceded by AT-rich sequences and activate transcription.

All three elements R1–R3 seem to be important for full activation of the promoter by RORα, since mutation of either element substantially reduced the activity. Deletion of the first element, however, was enough to almost completely abolish activity and deletion of R2 in tandem did not lead to a further reduction in activity. Activity mediated by RORα through the R2 site might thus be dependent on the R1 site and/or on other regulatory sequences located upstream of R1 to which other transcription factors could bind and interact with RORα. Using TESS (transcription element search software) to search for additional transcription factor binding sites, potential binding sites for HNF-1 and HNF-3 were found [53]. These or other transcription factors could potentially interact with RORα and be required for full activation of the promoter.

Deletion of all three AGGTCA motifs (R1–R3) reduces activity more than deleting only two motifs (R1–R2). In addition, mutation of the single R3 element reduces activity, implying a role for R3 in activation of the promoter. No reduction of RORα-induced activity is seen when mutating R4, which is in accordance with the binding-competition assay showing that RORα does not bind this element.

PPARα and RORα activate ApoAV transcription and have R2 as a common binding site. There may therefore be a crosstalk between the receptors in regulation of this gene either by the binding of both receptors simultaneously to the promoter or by competition for the R2 site. Differential activation of the ApoAV promoter by RORα or PPARα might be determined by different physiological conditions. PPARα is activated by fatty acids and fatty acid derivatives, whereas cholesterol has been suggested to be the endogenous ligand for RORα. Different metabolic conditions defined by different compositions of triglyceride and cholesterol in plasma might thus preferentially induce either PPARα- or RORα-dependent transcription of ApoAV.

A previously described polymorphism in the ApoAV promoter associated with triglyceride levels is located upstream of the promoter fragment analysed here [33]. Whether it influences ROR or PPAR dependent expression of the ApoAV gene remains to be elucidated.

Since ApoAV expression affects triglyceride levels, which have been shown to be an independent risk factor for coronary heart disease, developing drugs regulating ApoAV expression may be of clinical importance [33,36,54]. Fibrates, that activate PPARα, have been used therapeutically as lipid lowering drugs and have multiple effects on lipid metabolism [55]. So far, no potent synthetic activator of RORα has been identified, but if such a compound were developed, it could perhaps be used to lower triglyceride levels through activation of ApoAV transcription. However, RORα has also been shown to upregulate ApoCIII, which is positively associated with triglyceride levels [21]. The net outcome of treatment with an RORα agonist would therefore depend on a balance between the expression of ApoAV and ApoCIII. Regulation of ApoCIII expression is further complicated by the fact that Rev-erbα, that has been shown to reduce ApoCIII transcription, is also a target gene for both RORα and PPARα [5,10,31,56,57].

Several transcription factors and nuclear receptors have been shown to regulate apolipoprotein expression and there probably exists a complex network of transcriptional regulators to respond to the needs of an organism during different metabolic conditions. Our work suggests that RORα is one of these regulators possibly activated by cholesterol or by other means. Identification of a potent synthetic ligand for RORα would help to elucidate the role of RORα in metabolism and other physiological processes.

Acknowledgments

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