Transcriptional Regulation of Apolipoprotein A-I Gene Expression by the Nuclear Receptor RORα*

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Since elevated concentrations of plasma high density lipoprotein (HDL) and its major apolipoprotein (apo), apoA-I, confer protection against atherosclerosis, considerable research efforts have focused on the identification of factors regulating apoA-I gene expression in an attempt to increase its production. Nuclear receptors are interesting candidates because they are transcription factors whose activity is ligand-dependent. In the present study, we identified the orphan receptor RORα as an activator of apoA-I gene transcription. In apoA-I-expressing intestinal Caco-2 cells, overexpression of the RORα, but not the RORα2 or RORα3 isoforms, increased rat apoA-I gene transcription. Deletion and site-directed mutagenesis experiments identified a functional ROR-responsive element (RORE) in the rat and mouse apoA-I gene promoters, which overlaps with the TATA box. Gel shift experiments indicated that this RORE binds the RORα1 isoform, but not the RORα2 or RORα3 isoforms. Furthermore, compared with wild type mice, apoA-I mRNA levels were significantly lower in small intestines of staggerer mice homozygous for a deletion in the RORα gene. In addition, reverse transcriptase-polymerase chain reaction analysis revealed the expression of RORα in small intestinal epithelium and in Caco-2 cells. These data indicate a novel, physiological role for RORα1 in the regulation of genes involved in lipid and lipoprotein metabolism and possibly in the development of metabolic diseases, such as atherosclerosis.

Results from several epidemiological studies have demonstrated that plasma concentrations of high density lipoprotein (HDL) and its major protein component, apolipoprotein (apo) A-I, are inversely correlated to the development of coronary artery disease (1–4). In addition, studies in transgenic mice and rabbits have shown that overexpression of the human apoA-I gene results in increased plasma HDL and apoA-I concentrations and confers protection against atherogenesis (5, 6). Furthermore, elimination of the apoA-I gene by homologous recombination leads to a profound hypo-α-lipoproteinemia (7). Therefore, a thorough knowledge of the factors controlling apoA-I production and metabolism is essential to understand the causes of hypo-α-lipoproteinemia, the most common lipoprotein abnormality in patients with coronary artery disease (8).

To identify factors regulating apoA-I gene expression, we focussed our attention on various members of the superfamily of nuclear receptors. Nuclear receptors are transcription factors, which upon activation by specific ligands bind to response elements located in the regulatory regions of target genes and thereby modulate their transcriptional activity (9). As such nuclear receptors translate signals coming from the environment into changes in gene expression and are therefore interesting targets for pharmacological intervention. The largest class of nuclear receptors is constituted by the orphan receptors, for which no ligands have yet been identified (10). Furthermore, the physiological functions of most of the orphan receptors are not or only partly known, and target genes remain to be identified. Previous studies have shown that apoA-I gene expression is under control of various activators/ligands of nuclear receptors, such as glucocorticoid and thyroid hormones, estrogens, retinoids, and the hypolipidemic fibrate drugs, which are potent PPARα activators (11–15).

The ROR (retinoic acid receptor-related orphan receptor; also termed RZR) orphan receptors (16–18) constitute a subfamily of orphan receptors encoded by three different genes, RORα, RORβ, and RORγ (16, 18, 19). RORs bind as monomers to response elements consisting of a 6-bp AT-rich sequence preceding the half-core PuGGTCA motif (16, 20, 21). Due to alternative splicing and promoter usage, the RORα gene gives rise to 4 isoforms, α1, α2, α3, and RZRα (16–18), which differ in their N-terminal domains and display distinct DNA recognition and transactivation properties (16). Interestingly, a recent report identified the monogenic mutant staggerer (sg/sg) mice as deficient for RORα expression, due to a deletion in the gene of RORα (22). This deletion in the RORα gene in sg/sg mice prevents the translation of the putative ligand-binding domain, thereby presumably disrupting the normal function of this transcription factor (22). staggerer mice display a defect in the development of the Purkinje cells resulting in a severe neurological disorder characterized by cerebellar ataxia (23). However, at present it is unknown whether RORα deficiency in these mice also results in metabolic abnormalities.

In the present study, we identified RORα as a positive regulator of rat and mouse apoA-I gene transcription. Furthermore, we show that the expression of the apoA-I gene in the

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**Experimental Procedures**

Cloning and construction of recombinant plasmids—PCR amplification and cloning of the rat apoA-I gene promoter fragments into the pBLCAT5 promoterless expression vector were described previously (24). Site-directed mutagenesis of the RORE was accomplished using the oligonucleotide 5′-CAC ACA TAT ATA GGC AGG GAA GGA GA-3′ as a mutagenic primer on single-stranded DNA templates according to Kunkel (25). The rat wild-type (5′-GAT CCA CAC ATA TAT AGG TCA GGG AAG AAG A-3′), mutant (5′-GAT CCA CAC ATA TAT AGG CAG GGA AGA AGA-3′), and mouse (5′-GAT CCA CAC ATA TAT AGA CCA GGG AAG AAG A-3′) apoA-I RORE and the consensus RORE (5′-GAT CCA GGT TAG AAT GTA GGT CAA-3′) (20) oligonucleotides were cloned upstream of the thymidine kinase (TK) promoter of pBLCAT4 (26). Identity of all clones was verified by sequence analysis.

Transfections and transient expression assays—Mice were housed 2–5 per cage and maintained at 25 °C in a temperature-controlled room with a 12-h light-dark cycle. Water and food were given ad libitum. The small intestine was removed and divided into two sections of equal length (proximal and distal), opened longitudinally, washed in phosphate-buffered saline, and immediately frozen in liquid nitrogen. RNA extractions from rat and mouse intestinal epithelium were performed as described (28). The above described rat and mouse apoA-I and the consensus RORE double-stranded oligonucleotides (20) were end-labeled using T4 polynucleotide kinase and [γ-32P]ATP. For competition experiments, the indicated amounts of cold oligonucleotide were included just before labeled oligonucleotide. Each well was transfected with the same amount of DNA. CAT assays were performed exactly as described previously (27). A RSV-driven β-galactosidase expression vector (1 μg) was included as a control for transfection efficiency. Autoradiographs of CAT assays were quantified by scintillation counting, and results were normalized for transfection efficiency. Transfection experiments were performed in triplicate and represent at least 3 times.

**Gel Retardation Assays**—The pCMX-based RORα expression plasmids were in vitro transcribed using T7 polymerase and subsequently transfected using rabbit reticulocyte lysate as directed by the manufacturer (Promega). DNA-protein binding assays were conducted as described (28). The above described rat and mouse apoA-I and the consensus RORE double-stranded oligonucleotides (20) were end-labeled using T4 polynucleotide kinase and [γ-32P]ATP. For competition experiments, the indicated amounts of cold oligonucleotide were included just before labeled oligonucleotide was added.

Animal and RNA Analysis Experiments—Staggerer (sg/sg) mutant mice (10 weeks of age) were obtained by crossing known heterozygotes (+/sg) and identifying homozygous offspring by their clinical ataxia. Mice were housed 2–5 per cage and maintained at 25 °C in a temperature-controlled room with a 12-h light-dark cycle. Water and food were given ad libitum. The small intestine was removed and divided into two sections of equal length (proximal and distal), opened longitudinally, washed in phosphate-buffered saline, and immediately frozen in liquid nitrogen. RNA extractions from rat and mouse intestinal epithelium were performed as described (11). A-actin probe was used as a control probe (29).

For analysis of RORα expression, total RNA (100 ng) was reverse-transcribed using random hexamer primers and superscript reverse transcriptase. RORα mRNA was subsequently PCR-amplified (35 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C) using as primers the sense oligonucleotide 5′-GTC AGC AGC TTC TAC CTG GAC-3′ and the antisense oligonucleotide 5′-GTT TGG TTC TGG TGA GAG TGA AAG GCA CG-3′ which are conserved between human and mouse (16, 22). The amplified fragment spans the deleted region in mouse, rat, and human RORα coding sequence, yielding a fragment of the expected size of 482 and 359 bp in wild type and staggerer mice, respectively.

**Results and Discussion**

To determine whether apoA-I is a RORα target gene, the rat apoA-I gene promoter was cloned in front of a CAT reporter gene (22), under control of the CAT gene promoter. Transfection experiments were performed in CV-1 cells. The presence of a single copy of the rat apoA-I RORE, both cloned in the sense and antisense directions, resulted in a significant induction of TK promoter activity by apoA-I RORE (24). In contrast, transfection of pCMX plasmid alone did not activate apoA-I RORE (Fig. 3B). Interestingly, apoA-I overexpression consistently resulted in a more pronounced transactivation of the rat apoA-I RORE (cloned in its natural sense) compared with the previously described optimal RORE consensus sequence (Fig. 3A). A point mutation in the RORE resulting in a 7-fold increase of CAT activity driven by a 1-kilobase pair apoA-I gene promoter fragment (Fig. 1A). By contrast, neither the activities of the promoterless pBLCAT5 vector or a herpes simplex virus TK promoter-driven CAT vector were induced by RORα co-transfection (not shown), indicating that apoA-I promoter transactivation by RORα is specific. Upon 5′ deletion of the apoA-I promoter, basal promoter activity dropped significantly in Caco-2 cells, an observation which is concordant with the absence of intestinal expression of an apoA-I transgene driven by the proximal apoA-I promoter (30, 31). However, RORα overexpression still increased CAT activity approximately 4-fold (Fig. 1A). Since the apoA-I gene gives rise to different isoforms, we compared the transactivation potential of the RORα, -α2, and -α3 isoforms on the smaller rat apoA-I gene promoter construct next. In contrast to RORα1, neither RORα2 nor RORα3 could activate apoA-I gene transcription (Fig. 1B). These data indicate the presence of a ROR-response element (RORE) in the first 252 bp of the apoA-I gene promoter, which is specific for the RORα1 isoform. In vitro binding site selection experiments have indicated that RORα1 binds preferentially to a half-core AGGTCA nuclear receptor recognition motif preceded by an AT-rich sequence (16, 20). Although the apoA-I gene promoter A- and C-sites have been shown to bind different members of the nuclear receptor superfamily (28, 32–34), none of them are preceded by an AT-rich sequence. However, careful inspection of the apoA-I gene promoter revealed the presence between nucleotides −21 and −32 of a perfectly conserved AGGTCA half-site preceded by the sequence ATATAAT, which coincidently overlaps the TATA box (Fig. 2A). To determine whether this site mediates the effects of RORα1 on apoA-I gene transcription, we introduced a mutation in the AGGTCA half-core motif, which should inactivate the AGGTCA site without affecting the TATA box function (Fig. 2A). In contrast to the wild-type (wt) construct, the mutated (mt) construct was no longer activated by RORα1 overexpression in Caco-2 cells (Fig. 2B and C).

To determine whether the apoA-I RORE could confer RORα responsiveness to a heterologous promoter, the rat apoA-I RORE was cloned in front of the viral TK promoter, and co-transfection experiments were performed in CV-1 cells. The presence of a single copy of the rat apoA-I RORE, both cloned in the sense and antisense directions, resulted in a significant induction of TK promoter activity by apoA-I RORE (24). In contrast, transfection of pCMX plasmid alone did not activate apoA-I RORE (Fig. 3B). Interestingly, apoA-I overexpression consistently resulted in a more pronounced transactivation of the rat apoA-I RORE (cloned in its natural sense) compared with the previously described optimal RORE consensus sequence (Fig. 3A). A point mutation in the RORE resulting in a 7-fold increase of CAT activity driven by a 1-kilobase pair apoA-I gene promoter fragment (Fig. 1A). By contrast, neither the activities of the promoterless pBLCAT5 vector or a herpes simplex virus TK promoter-driven CAT vector were induced by RORα co-transfection (not shown), indicating that apoA-I promoter transactivation by RORα is specific. Upon 5′ deletion of the apoA-I promoter,
the AT-rich motif is conserved between the rat and mouse apoA-I gene promoters, two nucleotide differences occur in the AGGTCA motif (Fig. 3A). To determine whether this mouse apoA-I site could function as a RORE, we cloned three copies of the putative mouse apoA-I RORE in front of the TK promoter and performed co-transfection experiments with RORα in CV-1 cells. Although less pronounced compared with the rat apoA-I RORE, overexpression of RORα resulted in a significant activation of the TK promoter construct driven by three copies of the mouse apoA-I RORE (Fig. 3C).

Next, the binding activity of RORα to the mouse and rat apoA-I ROREs was determined by gel shift analysis and compared with that of the previously identified consensus RORE (20). RORα, but not RORα2 or RORα3, bound to the rat apoA-I RORE (Fig. 4, A and B), results which correlate well with the relative transactivation potential of the different RORα isoforms. This binding was specific since it could be competed by excess unlabeled oligonucleotide (Fig. 4A). Interestingly, the rat apoA-I RORE appears to bind RORα1 with higher affinity than the consensus RORE (Fig. 4A). Furthermore, RORα1 clearly binds to the mouse apoA-I RORE (Fig. 4C), although the affinity of the mouse RORE for RORα1 is clearly lower than that of the rat apoA-I RORE, a fact which correlates well with the relative transactivation of both ROREs by RORα1 (Fig. 3, B and C). These data indicate therefore that although the mouse apoA-I RORE appears to be a lower affinity RORE compared with the rat apoA-I RORE, it may be a functional RORE.

These observations prompted us to analyze the physiological role of RORα in the regulation of apoA-I gene expression. Since homozygous staggerer mice have been shown to be monogenic mutants for the RORα gene (22), the expression of the apoA-I gene in different parts of the small intestine of these mice was analyzed next. ApoA-I mRNA levels were significantly lower both in proximal and distal small intestines of staggerer mice compared with wild type mice (Fig. 5). As a control, β-actin mRNA levels were similar between sgsag and wild-type mice.

Finally, since direct regulation of apoA-I expression by RORα requires its expression in the small intestine, reverse transcriptase-PCR experiments were performed on RNA isolated from small intestinal epithelium cells from mice and rats as well as from Caco-2 cells. Using primers complementary to conserved sequences between mouse and human RORα (16, 22), a cDNA fragment of the expected size of 482 bp was amplified in rat and mouse epithelial cells as well as in Caco-2 cells, thereby indicating that RORα is expressed in the intestine (Fig. 6). Interestingly, in staggerer mice a truncated cDNA fragment of 359 bp exactly corresponding to the size of the deletion identified in the RORα gene in these mice was amplified (22). These observations warrant further studies in homozygous staggerer mice to determine the role of RORα in the control of HDL metabolism and its possible consequences for atherosclerosis susceptibility in vivo in staggerer mice.

Altogether these results clearly indicate that apoA-I is a direct target gene for RORα1 and that its expression in the intestine is under control of RORα1. To our knowledge this is the first RORα target gene with a function in lipid and lipoprotein metabolism. The selective transactivation and binding of RORα1, but not RORα2 or RORα3, to the apoA-I RORE is in
and wild type (+/+), mice, and apo-A-I and β-actin mRNA levels were measured as described under "Experimental Procedures."

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In conclusion, we have shown that both the mouse and rat apo-A-I genes are direct targets for RORα1 and that RORα plays a physiological role in the expression of the apo-A-I gene in the intestine. This is the first identification of a RORα target gene which is involved in plasma lipid and lipoprotein metabolism. These data suggest a physiological role of the nuclear receptor RORα in the control of lipoprotein metabolism.

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