Retinoids Increase Human Apo C-III Expression at the Transcriptional Level via the Retinoid X Receptor

Contribution to the Hypertriglyceridemic Action of Retinoids

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Abstract

Hypertriglyceridemia is a metabolic complication of retinoid therapy. In this study, we analyzed whether retinoids increase the expression of apo C-III, an antagonist of plasma triglyceride catabolism. In men, isotretinoin treatment (80 mg/d; 5 d) resulted in elevated plasma apo C-III, but not apo E concentrations. In human hepatoma HepG2 cells, retinoids increased apo C-III mRNA and protein production. Transient transfection experiments indicated that retinoids increase apo C-III expression at the transcriptional level. This increased apo C-III transcription is mediated by the retinoid X receptor (RXR), since LG1069 (4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphtalenyl)ethenyl]benzoic acid), a RXR-specific agonist, but not TTNPB ((*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtalenyl)propenyl]benzoic acid), a retinoic acid receptor (RAR)-specific agonist, induced apo C-III mRNA in HepG2 cells and primary human hepatocytes. Mutagenesis experiments localized the retinoid responsiveness to a *cis*-element consisting of two imperfect AGGTCA sequences spaced by one oligonucleotide (DR-1), within the previously identified C3P footprint site. Cotransfection assays showed that RXR, but not RAR, activates apo C-III transcription through this element either as a homo- or as a heterodimer with the peroxisome proliferator–activated receptor. Thus, apo C-III is a target gene for retinoids acting via RXR. Increased apo C-III expression may contribute to the hypertriglyceridemia and atherogenic lipoprotein profile observed after retinoid therapy. (*J. Clin. Invest.* 1998. 102:625–632.) Key words: gene regulation • triglycerides • hyperlipidemia • retinoids • nuclear receptors

Introduction

Retinoids are vitamin A derivatives used for the treatment of vitamin A deficiency and dermatological disorders, as well as chemoprophylaxis and therapy of certain cancers (1). Retinoids act by modulating the transcription of target genes, thereby interfering with cellular regulation, growth, and differentiation processes. Retinoids are ligands for transcription factors of the nuclear receptor superfamily (2). Two distinct classes of retinoid receptors exist: the retinoic acid receptors (RAR)[1] and the retinoid X receptors (RXR). Whereas 9-*cis* retinoic acid (RA; 9cRA) is a natural ligand for both RAR and RXR, all-*trans* RA (tretinoin) (atRA) is selective for RAR. Since natural RA isomers are unstable leading to interconversion in vivo, stable RAR- and RXR-specific ligands, such as TTNPB ((*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtalenyl)propenyl]benzoic acid) and LG1069 (4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphtalenyl)ethenyl]benzoic acid) respectively, have been synthesized, allowing specific targetting of the RAR and RXR signal transduction pathways (3, 4). RAR heterodimerizes with RXR and binds to specific response elements (RARE) composed of two degenerated AGGTCA sequences oriented as direct repeats separated by two or five nucleotides (DR-2 or DR-5). RXR also activates transcription either as homo- or as heterodimer with peroxisome proliferator–activated receptor (PPAR) from DR-1 elements (RXRE). In addition to its teratogenicity, the most frequent systemic side effect of RA administration is an increase in plasma triglycerides, with one out of five patients developing overt hypertriglyceridemia (5). The mechanism behind this RA-induced hypertriglyceridemia is, however, unknown.

Apo C-III is a key player in plasma triglyceride metabolism. Plasma apo C-III concentrations are positively correlated with plasma triglycerides, both in normal and hypertriglyceridemic subjects (6–8). Several lines of evidence causally link apo C-III with plasma triglyceride catabolism. First, apo C-III deficiency results in increased VLDL catabolism, whereas increased apo C-III synthesis occurs in hypertriglyceridemic patients (9, 10). Second, an association exists between certain apo C-III gene polymorphisms and increased plasma apo C-III and triglyceride concentrations (11, 12). Finally, overexpression of human apo C-III in mice resulted in hypertriglyceri-

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Received for publication 22 August 1997 and accepted in revised form 1 June 1998.

J. Clin. Invest.

0021-9738/98/08/0625/08 $2.00
Volume 102, Number 3, August 1998, 625–632
http://www.jci.org

1. *Abbreviations used in this paper:* atRA, all-*trans* retinoic acid; CAT, chloramphenicol acetyl transferase; DR-1, DR-2, DR-5, direct repeat spaced by one, two, and five nucleotides, respectively; LG1069, (4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphtalenyl)ethenyl]benzoic acid); LPL, lipoprotein lipase; 9cRA, 9-*cis* retinoic acid; PPAR, peroxisome proliferator–activated receptor; RXR, retinoid X receptor; RXRE, retinoid X receptor response element; TK, thymidine kinase; TTNPB, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtalenyl)propenyl]benzoic acid; WT, wild-type.

demia, whereas elimination of the endogenous apo C-III gene results in lowered plasma apo C-III concentrations and protects the animals against postprandial hypertriglyceridemia (13, 14). Apo C-III acts by delaying the catabolism of triglyceride-rich particles by inhibiting their binding to the endothelial surface and lipolysis by lipoprotein lipase (LPL) as well as by interfering with apo E-mediated receptor clearance of remnant particles from plasma (15–19).

In this study, we demonstrate that retinoids increase plasma apo C-III concentrations by inducing hepatic apo C-III gene expression at the transcriptional level via a RXR-mediated mechanism. These data indicate that retinoids may increase plasma triglyceride concentrations, at least in part, by stimulating apo C-III gene expression.

Methods

Clinical protocol. A randomized, double-blind, placebo-controlled, crossover study, approved by the local Ethics Committee, was conducted at the Center for Human Drug Research, University Hospital Leiden, The Netherlands with 21–26 yr-old healthy male volunteers with isotretinoin (80 mg/d for 5 d) (20). After a 2-wk washout period, subjects were given the alternative treatment. The volunteers abstained from other drugs, alcohol, and caffeine-containing beverages and from strenuous physical activities 24–48 h before the start as well as during the entire study period. On the mornings before and after the treatment period, fasting blood was collected and plasma was separated by centrifugation and stored at −80°C until analysis. The Wilcoxon signed rank test was used to compare biological variables between patients who had received either the retinoid or the placebo treatment.

Lipid, apo C-III, and apo E measurements. Plasma triglycerides (triglycerides GPO-PAP; Boehringer Mannheim, Mannheim, Germany) were determined enzymatically. ApoE (21) and apo C-III (22) in plasma and culture media were measured by a noncompetitive sandwich ELISA.

Cell culture and RNA analysis. Human hepatoma HepG2 and HeLa cells were maintained in DME supplemented with 10% FCS at 37°C in 5% CO_2/95% O_2. Human liver specimens were collected from healthy multiorgan donors for transplantation who died after severe traumatic brain injury at the Moscow Medical Center. Permission to use nontransplanted parts of donor liver for scientific research was obtained from the Russian Ministry of Health. Hepatocytes were obtained by a two-step collagenase perfusion (23). Cells were resuspended in DME with Earl's salts with 10% FCS, 2 mM L-glutamine, and 50 mg/ml gentamycin, seeded at a density of 1.5×10^5 cells/cm^2 on rat-tail type I collagen-coated dishes, and incubated in 5% CO_2/95% air at 37°C. The medium was renewed after a 4-h adhesion period and treatments were started after 20 h.

For experiments, cells were changed to medium containing 10% FCS delipoproteinized by ultracentrifugation in KBr (1.21 g/ml) and treated with AG-1-X8 resin plus activated charcoal. Stimuli were dissolved in DMSO (0.1% vol/vol) and added to the medium at the indicated concentrations and periods of time. The control cells received vehicle only.

RNA extractions and Northern and dot blot hybridizations of apo C-III mRNA were performed as described (23). Autoradiograms were analyzed with a quantitative scanning densitometer (GS670; Bio-Rad Laboratories, Fullerton, CA) and results normalized to human ribosomal protein 36B4 mRNA levels (23, 24).

Apo C-III immunoprecipitation experiments. HepG2 cells were cultured in DME containing 0.2% BSA and fenofibric acid (5×10^{-4} M), LG1069 (10^{-6} M) or vehicle (DMSO) for 24 h, washed in PBS, and incubated in methionine-free medium with ^{35}S-labeled methionine (0.02 mCi/ml) for 5 h. Cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% [wt/vol] sodium deoxycholate, 1% [vol/vol] Triton X-100, 0.25% [wt/vol] SDS). After centrifugation (10,000 *g*), cell lysates were incubated overnight at 4°C with polyclonal anti-apo C-III antibody in RIPA buffer. Immunoprecipitates were collected using protein A-Sepharose, washed four times in RIPA buffer, boiled in SDS-PAGE loading buffer (50 mM Tris, pH 7.5, 2.5% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, 10% [vol/vol] glycerol) and subsequently separated by 15% SDS-PAGE. After drying, gels were exposed onto BIOMAX-MS films (Eastman Kodak, Rochester, NY).

Construction of recombinant plasmids. The −1415/+24 genomic human apo C-III gene promoter, prepared by EcoRI/PvuII digestion, blunt-ended and cloned in the HincII site of pUC18, was excised by HindIII and BamHI digestion and subcloned in the corresponding restriction sites of the pBLCAT5 expression vector generating plasmid −1415/+24CIII-CAT. Site-directed mutagenesis of the RXRE in the apo C-III promoter C3P footprint (25) was accomplished by the method of Nakayame and Eckstein (26) using an oligonucleotide spanning the apo C-III promoter −58/−80 region containing two mutations (5′-TGA CCT TTG CTG AGC GCC CTG GG-3′) to generate −1415/C3P$_{mt}$/+24CIII-CAT. The wild-type (WT) and mutated −198/+24 apo C-III promoter vectors were obtained by PstI digestion and religation of the corresponding −1415/+24CIII-CAT vectors.

The C3P$_{wt}$ and C3P$_{mt}$ oligonucleotides (bases −62/−88; 5′-gatc TCA GCA GGT GAC CTT TGC CCA GCG CCC g-3′ and 5′-gatc TCA GCA GGT GAC CTT TGC TGA GCG CCC g-3′) were cloned into the BamHI/BglII sites of pIC20H (27), HindIII digested and subcloned upstream of the thymidine kinase (TK) promoter in pBLCAT4 generating (C3P$_{wt}$)$_2$-TKCAT, (C3P$_{mt}$)$_2$-TKCAT and (C3P$_{wt}$)$_4$-TKCAT (containing two, two, and four copies of the oligonucleotides, respectively).

Transient transfection assays. pSG5mRXRα and pSGmRARα were the kind gifts of P. Chambon (28). Transfections were performed at 50–60% confluency by the calcium phosphate coprecipitation procedure with a mixture of plasmids containing the CAT reporter (1–5 μg/60-mm dish) and expression vector(s) (200 ng). A CMV-driven β-gal expression vector (0.35 μg) was used as a control for transfection efficiency (29). All samples were complemented with pSG5 plasmid to an equal total amount of DNA. After transfection cells were incubated for 40 h with the indicated compounds or vehicle (DMSO) in medium containing 5% delipoproteinized calf serum treated with AG-1-X8 resin plus activated charcoal. CAT and β-gal assays were performed on cell extracts (23).

Results

Isotretinoin treatment increases plasma apo C-III concentrations. Administration of isotretinoin, the 13-*cis* RA isomer of atRA which is used for oral treatment of dermatological disorders, has been shown to increase plasma triglycerides in hu-

Table I. Influence of Isotretinoin Treatment on Plasma Triglyceride, Apo C-III, and Apo E Concentrations

	Placebo	Isotretinoin	*P*
Triglycerides (mg/dl)	75±29	103±34	< 0.03
Apo C-III (mg/dl)	1.64±0.52	2.03±0.54	< 0.02
Apo E (mg/dl)	3.07±0.43	2.88±0.56	NS

Isotretinoin (80 mg/d for 5 d) was given to nine healthy, male volunteers as described in Methods. Plasma lipid and apo concentrations were measured and values are expressed as means±SD. Differences between placebo and isotretinoin groups were compared and *P* values calculated using a paired Wilcoxon signed rank test.

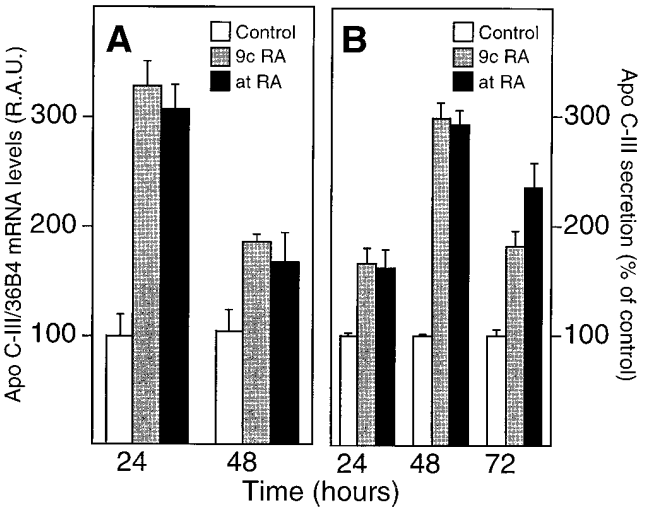

Figure 1. All-*trans* and 9-*cis* retinoic acids increase apo C-III mRNA levels (*A*) and protein secretion (*B*) in human hepatoma HepG2 cells. HepG2 cells were treated with 10 μM of 9cRA, atRA, or vehicle (DMSO) for the indicated periods of time and apo C-III mRNA levels (*A*) and protein secretion (*B*) were determined as described in Methods. Apo C-III mRNA levels are normalized to 36B4 control mRNA levels and expressed in relative arbitrary units (RAU). All values are expressed as mean±SD of three points.

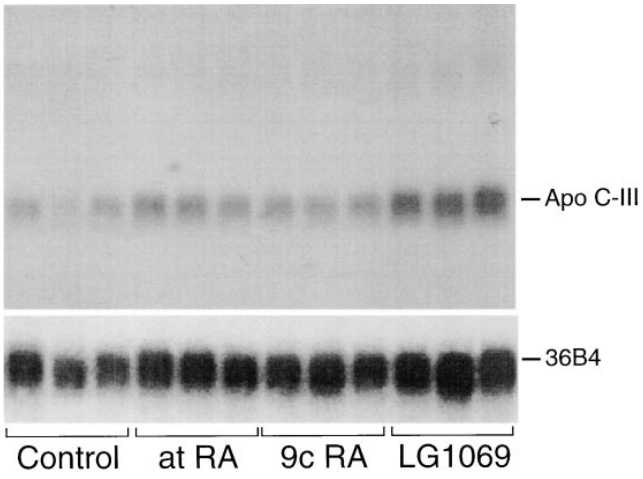

Figure 3. Retinoids increase apo C-III mRNA levels in primary cultures of adult human hepatocytes. Human hepatocytes were isolated and treated for 24 h with 10 μM of the indicated retinoids or vehicle (DMSO; *Control*). 10 μg of total RNA was subjected to electrophoresis, transferred to a nylon membrane, and hybridized to apo C-III (*top*) or 36B4 (*bottom*) cDNA probes as described in Methods.

mans (5). To investigate the influence of isotretinoin treatment on apo C-III, volunteers were treated for 5 d with isotretinoin (80 mg/d) and plasma concentrations of triglycerides, apo C-III, and apo E were measured. Isotretinoin treatment significantly increased plasma triglycerides and apo C-III concentrations (Table I). By contrast, plasma apo E concentrations were not affected by isotretinoin treatment (Table I).

Retinoids increase apo C-III gene expression and protein secretion in human hepatoma HepG2 cells. To determine whether the increase in plasma apo C-III concentrations after retinoid treatment is due to an increased expression of apo C-III in liver, human hepatoma HepG2 cells were incubated with atRA, 9cRA, or vehicle and apo C-III mRNA levels and protein secretion in the medium were analyzed. Apo C-III mRNA levels increased significantly both after 24 and 48 h of 9cRA or atRA addition, attaining more than threefold induction after 24 h (Fig. 1 *A*). This elevation of apo C-III mRNA levels was accompanied by an increased secretion of apo C-III protein in the medium, attaining a threefold increase after 48 h (Fig. 1 *B*).

By contrast, apo E secretion did not change upon retinoid treatment (data not shown).

RXR-, but not RAR-specific retinoids increase apo C-III expression in human primary hepatocytes and hepatoma HepG2 cells. Since the different RA isomers are unstable and substantial interconversion occurs, the analysis of the effects of atRA and 9cRA does not allow one to distinguish the relative involvement of the RAR and RXR signal transduction pathways in the activation of apo C-III expression by retinoids. To determine whether the effects of retinoids on apo C-III gene expression are mediated through RXR or RAR, experiments were performed using the synthetic RXR- and RAR-specific agonists LG1069 and TTNPB (3, 4). In HepG2 cells, both 9cRA and atRA induced apo C-III mRNA levels in a dose-dependent manner, increasing already at concentrations between 10^{-9} and 10^{-8} M (Fig. 2). The RXR agonist LG1069 also increased apo C-III mRNA levels in a dose-dependent manner, although the effect was less pronounced compared to 9cRA (Fig. 2). By contrast, the RAR agonist TTNPB did not increase, and, at higher doses (10^{-6}–10^{-5} M), rather decreased apo C-III mRNA (Fig. 2). In primary human hepatocytes, both atRA, 9cRA, and LG1069 increased apo C-III mRNA, with

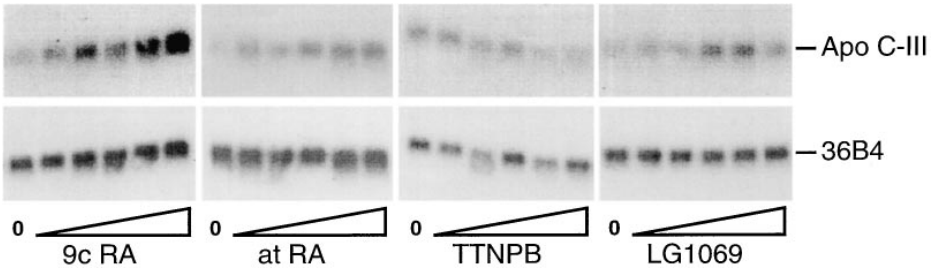

Figure 2. The RXR-, but not RAR-specific retinoids increase apo C-III mRNA levels in human hepatoma HepG2 cells. HepG2 human hepatoblastoma cells were treated for 24 h with vehicle (DMSO; 0) or increasing concentrations (10^{-9}–10^{-5} M) of 9cRA, atRA, LG1069, or TTNPB. RNA was prepared, 10 μg of total RNA was subjected to electrophoresis, transferred to a nylon membrane, and hybridized to apo C-III (*top*) or 36B4 (*bottom*) cDNA probes as described in Methods.

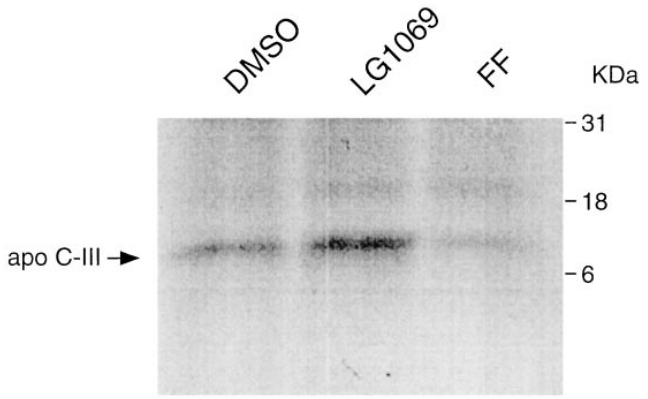

Figure 4. Retinoids increase apo C-III protein synthesis in human hepatoma HepG2 cells. HepG2 cells were treated for 24 h with 1 μM LG1069, 500 μM fenofibric acid (*FF*) or vehicle (*DMSO*), labeled with [^{35}S]methionine for 5 h and apo C-III was immunoprecipitated from whole cell extracts and separated by SDS-PAGE as described in Methods.

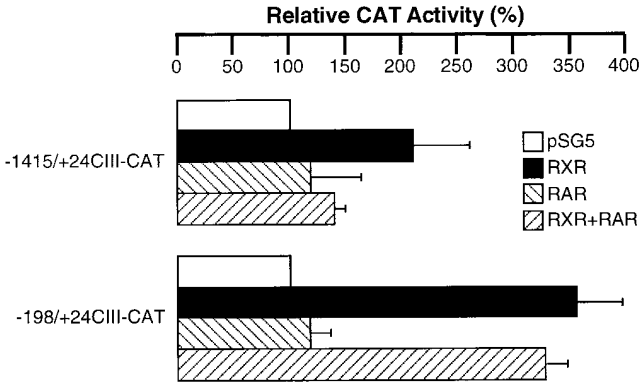

Figure 5. RXR, but not RAR, induces apo C-III gene expression at the transcriptional level via the apo C-III promoter. HepG2 cells were transiently transfected with the indicated human apo C-III gene promoter containing CAT plasmids in the presence of cotransfected pSG5 control vector, mRXRα, mRARα, or mRXRα and mRARα together, and treated for 40 h with 1 μM of 9cRA. CAT activity was measured and expressed (mean±SD) as described in Methods.

the effect of LG1069 being most pronounced (control: 100±38; 9cRA: 197±17; atRA: 239±37; LG1069: 465±67; all $P < 0.05$ from control) (Fig. 3). These data indicate that the induction of apo C-III expression is mediated via activation of RXR, but not RAR. The observation that TTNPB may repress apo C-III expression is congruent with this and previous data showing a negative transcriptional activity of RAR on RXR DR-1 response elements (30, 31).

To analyze whether the increase of apo C-III expression after retinoids is parallelled by enhanced de novo protein synthesis, [^{35}S]methionine incorporation experiments were performed. HepG2 cells were treated for 24 h with LG1069 or vehicle, labeled with [^{35}S]methionine and apo C-III protein subsequently immunoprecipitated from cell extracts using a human apo C-III specific polyclonal antibody. LG1069 increased [^{35}S]methionine incorporation in apo C-III protein. By contrast, fenofibric acid, a PPARα activator that has been shown to decrease apo C-III expression (23), lowered apo C-III synthesis (Fig. 4). These data indicate that the RXR agonist LG1069 increases de novo apo C-III protein synthesis in HepG2 cells.

Retinoids increase apo C-III expression at the transcriptional level. To determine whether retinoids increase apo C-III expression at the transcriptional level, HepG2 cells were transiently transfected with a CAT expression vector driven by the human apo C-III promoter containing the liver-specific enhancer elements, in the presence of mRXRα, mRARα, or both and cells were treated with 9cRA. mRXRα significantly increased apo C-III promoter activity, whereas mRARα, whether alone or in the presence of mRXRα, did not affect apo C-III transcription (Fig. 5). When a unilateral deletion construct containing bp −198/+24 of the apo C-III promoter was transfected into HepG2 cells RXR-specific transcriptional induction was preserved, demonstrating the presence of a RXR-response element within the proximal apo C-III promoter (Fig. 5).

To further explore the relative contributions of RAR and RXR, cotransfections were performed on the −1415/+24 apo C-III promoter vector in the presence of different retinoids. mRXRα cotransfection activated apo C-III promoter activity in the presence of atRA, 9cRA, and LG1069, but not TTNPB (Fig. 6). In contrast, mRARα in the presence of the same activators did not influence apo C-III promoter activity (data not shown). These results further indicate that retinoid induction of apo C-III gene expression is primarily mediated by RXR.

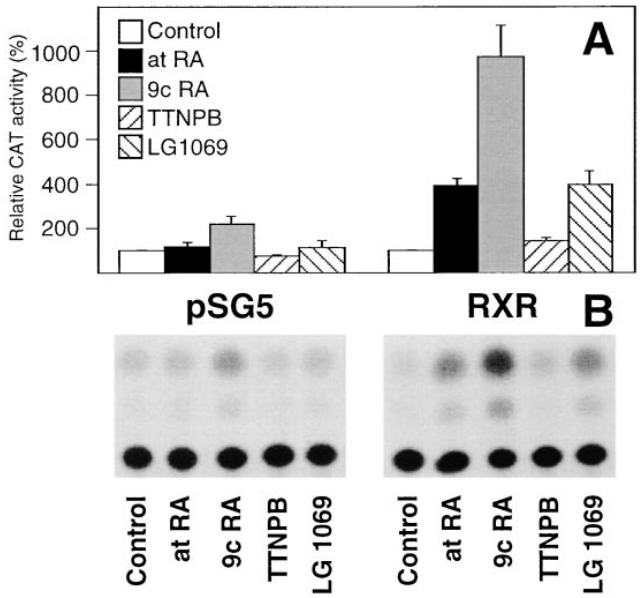

Figure 6. RXR, but not RAR agonists activate human apo C-III gene promoter transcription. (*A*) HeLa cells were cotransfected with the −1415/+24CIII-CAT plasmid in the presence of mRXRα or pSG5 vector plasmids. Cells were treated with atRA (1 μM), 9cRA (1 μM), LG1069 (1 μM), TTNPB (1 μM), or vehicle (DMSO; *Control*); CAT activity was measured and expressed as described in Methods. (*B*) CAT assay demonstrating the effects of atRA (1 μM), 9cRA (1 μM), LG1069 (1 μM), TTNPB (1 μM), or vehicle (DMSO; *Control*) on the expression of the −1415/+24CIII-CAT plasmid in the presence cotransfected mRXRα or empty pSG5 expression vector in CV-1 cells.

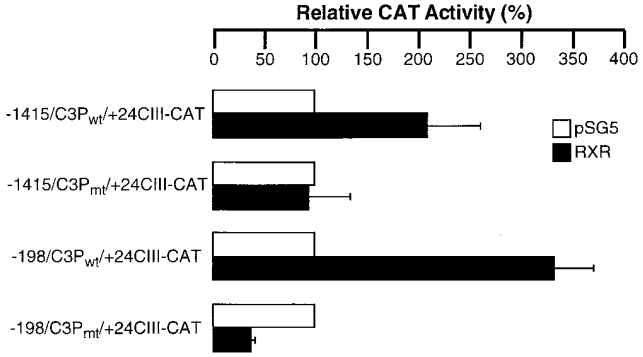

Figure 7. The induction of apo C-III gene transcription by RXR is mediated by a RXR response element localized in the C3P site of the apo C-III promoter. Site-directed mutagenesis of the putative DR-1 RXRE in the human apo C-III gene promoter C3P site was performed as described in Methods and HepG2 cells were transiently transfected with the wild-type (−1415/C3P$_{wt}$/+24CIII-CAT and −198/C3P$_{wt}$/+24CIII-CAT) or mutant (−1415/C3P$_{mt}$/+24CIII-CAT and −198/C3P$_{mt}$/+24CIII-CAT) apo C-III promoter-containing CAT vectors in the presence of cotransfected mRXRα or pSG5 vector plasmids in the presence of 9cRA (1 μM) and CAT activity was measured and expressed as described in Methods.

Identification of a RXRE in the apo C-III promoter. Sequence analysis of the −198/+24 apo C-III region indicated a potential RXRE composed of a DR-1 sequence within the C3P footprint site, which is essential for liver apo C-III transcription (25, 32, 33). To determine whether this DR-1 site mediates the retinoid response of apo C-III, two-point mutations were introduced in the putative RXR-responsive DR-1 sequence both in the −1415/+24 and the −198/+24 apo C-III promoter constructs. The effects of RXR and 9cRA on these mutated apo C-III promoter constructs were then analyzed. Whereas both the −1415/+24 and the −198/+24 WT promoters were significantly activated by mRXRα, mutation of the DR-1 sequence abolished its induction by RXR (Fig. 7), thereby indicating that the DR-1 sequence within the C3P footprint mediates RXR responsiveness of the apo C-III promoter.

To determine whether the apo C-III RXRE could confer retinoid responsiveness to a heterologous promoter, the WT and mutated C3P oligonucleotides were cloned in front of the TK promoter and cotransfections were performed in HeLa cells. Two copies of the WT, but not of the mutated C3P site, conferred induction of TK promoter activity by mRXRα (Fig. 8 *A*). Overexpression of mRARα activated neither WT nor mutated C3P sites and simultaneous expression of both mRXRα and mRARα resulted in intermediary transcriptional activation (Fig. 8 *A*), suggesting competition between transcriptionally active RXR homodimers and inactive RAR/RXR heterodimers. Finally, the effects of retinoids on the activation of the C3P site was analyzed in HepG2 cells. Again, only cotransfection with mRXRα, but not with mRARα, increased chloramphenicol acetyl transferase activity, which was further enhanced in the presence of atRA, 9cRA, and LG1069, but not by TTNPB (Fig. 8 *B*).

RXR activates apo C-III promoter activity either as a homo- or as a heterodimer with PPARα. Since the apo C-III promoter RXRE is a DR-1 sequence, to which not only RXR homo-, but also RXR/PPAR heterodimers can bind, the influence of PPARα on the RXR-mediated activation of apo C-III promoter transcription was analyzed. Therefore, the -1415/+24 apo C-III promoter was transfected in Cos cells with mRXRα, mPPARα, or both, and cells were treated with 9cRA or the PPARα ligand Wy14643 (34). Addition of 9cRA or Wy14643 did not influence WT apo C-III promoter activity (Fig. 9 *A*), suggesting that, under these conditions, Cos-1 cells do not express endogenous RXR or PPARα protein. In the presence of exogenous RXR, 9cRA induced apo C-III promoter activity sixfold, whereas Wy14643 did not influence its activity and Wy14643 together with 9cRA induced its activity to similar levels as 9cRA alone (Fig. 9 *A*), indicating that 9cRA most likely acts via RXR homodimers under these conditions. In the presence of PPARα, Wy14643 increased chloramphenicol acetyl transferase activity fourfold, whereas 9cRA was inactive and Wy14643 together with 9c RA induced its activity to similar levels as Wy14643 alone (Fig. 9 *A*). When both PPARα and RXR were cotransfected, a twofold activation of apo C-III promoter activity was observed in the absence of their ligands and a further fourfold activation was observed when cells were treated with either 9cRA, Wy14643, or both (Fig. 9 *A*). By contrast, the apo C-III promoter mutated in the DR-1 site was not activated under any of these conditions (Fig. 9 *B*). Identi-

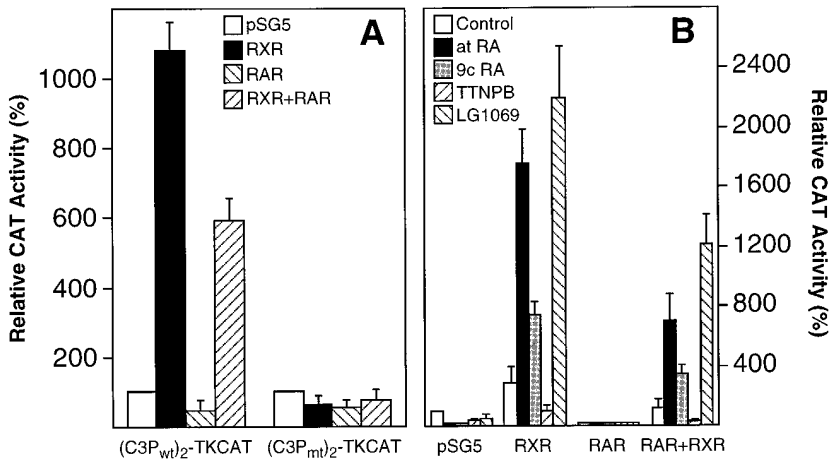

Figure 8. The apo C-III gene promoter C3P site confers RXR responsiveness to a heterologous TK promoter. The WT (C3P$_{wt}$) and mutated (C3P$_{mt}$) C3P oligonucleotides were cloned in front of the TK promoter as described in Methods. HeLa (*A*) or HepG2 (*B*) cells were transfected, respectively, with the (C3P$_{wt}$)$_2$-TKCAT and (C3P$_{mt}$)$_2$-TKCAT or the (C3P$_{wt}$)$_4$-TKCAT reporter vectors in the presence of mRXRα, mRARα, mRARα and mRXRα together, or pSG5 vector plasmids. Cells were treated with 9cRA (1 μM; *A*) or atRA (1 μM), 9cRA (1 μM), TTNPB (1 μM), LG1069 (1 μM), or vehicle (DMSO; *Control*) (*B*); CAT activity was measured and expressed as described in Methods.

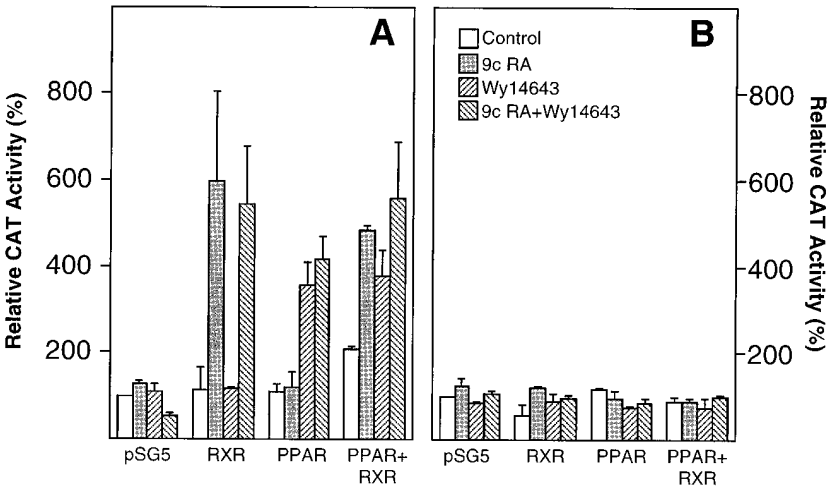

Figure 9. Both PPAR and RXR activate apo C-III gene promoter transcription via the C3P site. Cos cells were transiently transfected with the WT −1415/$C3P_{wt}$/+24CIII-CAT or mutant −1415/$C3P_{mt}$/+24CIII-CAT apo C-III promoter-containing CAT vectors in the presence of cotransfected mRXRα, hPPARα, both or pSG5 vector plasmids in the presence of 9cRA (1 μM), Wy14643 (1 μM), both, or vehicle (DMSO). CAT activity was measured and expressed as described in Methods.

cal results were obtained in cotransfection experiments in HepG2 cells on the $(C3P_{wt})_4$-TKCAT vector (data not shown). Thus RXR can activate apo C-III transcription from the C3P DR-1 site either as a homo- or as a heterodimer with PPAR.

Discussion

It is well-established that retinoids at pharmacological doses for the treatment of dermatological disorders increase plasma triglycerides, with ±20% of the patients developing an overt hypertriglyceridemia (> 200 mg/dl) (5). Here, we demonstrate that the elevation of plasma triglycerides after isotretinoin treatment is associated with increased levels of apo C-III, an antagonist of plasma triglyceride metabolism. By contrast, plasma concentrations of apo E, which mediates the clearance of triglyceride-rich lipoprotein particles from plasma, did not change after retinoid treatment, data which are in line with a previous report (35). This increase of apo C-III may explain the delayed clearance of triglyceride-rich lipoproteins observed in humans treated with retinoids (36, 37).

Retinoids act by increasing liver apo C-III expression, production, and secretion, indicating that apo C-III is a retinoid-response gene. By the use of RXR- and RAR-specific agonists, it is demonstrated that the induction of apo C-III transcription requires RXR, but not RAR activation. Furthermore, a DR-1-like RXRE was identified in the apo C-III promoter C3P site. Via this C3P site the nuclear receptor HNF-4 drives hepatic apo C-III gene expression (25, 32, 33). Furthermore, overexpression of a dominant negative form of HNF-4 results in a significant reduction of apo C-III gene expression in HepG2 cells (38). Our data indicate that RXR can act as a positive factor to further enhance apo C-III gene transcription above HNF-4-driven basal levels.

Apo C-III gene transcription is also negatively controlled via the C3P site through binding of nuclear receptors displaying negative transcriptional activity, such as COUP-TFI/Ear-3, COUP-TFII/Arp-1, and Ear-2 (33, 39). Interestingly, the hypotriglyceridemic action of fibrates is partly mediated via a repression of liver apo C-III gene transcription (23). Fibrates are ligands for PPARα, which binds to DR-1 elements as a heterodimer with RXR (40). The role of PPARα in the negative regulation of apo C-III gene expression by fibrates is demonstrated in PPARα knockout mice, in which apo C-III repression by fibrates is abolished (41). Interestingly, a PPAR-binding site has been localized in the apo C-III promoter C3P site (42). The repression of apo C-III transcription by fibrates is suggested to be due to both a reduction of HNF-4 expression as well as to competition for binding of the transcriptionally less active PPAR/RXR complex with the strong HNF-4 homodimer to this C3P site (42). In this report, we map this PPAR response element to the C3P DR-1 site footprint, coinciding with the retinoid-response element. Furthermore, transfection experiments in Cos and HepG2 cells, which express HNF-4 endogenously (38), indicate that retinoids activate apo C-III transcription via this element either as RXR homo- or as PPAR/RXR heterodimers. Thus, and like other positive PPAR response elements, the C3P DR-1 site can function as a positive PPRE. Since, and in contrast to retinoids, fibrates lower apo C-III gene expression (23) and protein synthesis (as shown in this study), our results suggest that the negative fibrate response element does not localize to the C3P site, but resides elsewhere in the apo C-III promoter. Since RXR-agonists increase apo C-III transcription, this negative fibrate-response element is unlikely to be a classical DR-1 site, in which RXR is required for PPAR binding and activity.

The results from this study add apo C-III to the list of genes involved in lipoprotein metabolism, whose expression is controlled by retinoids. Positive retinoid-response elements have been identified in the promoters of apo A-I and apo A-II, the major HDL apolipoproteins (31, 43). Positive RAR/RXR DR-2 and RXR/RXR DR-1 sites have been identified in the apo A-I promoter A footprint (43, 44). Furthermore, retinoids increase apo A-I mRNA levels and secretion in human, monkey, and rat hepatocytes (45–47). Similarly, a RXRE has been localized in the apo A-II promoter J-site, which mediates the induction of apo A-II expression in human hepatocytes (31). However, the physiological consequence of the effects of retinoids on the expression of these HDL apolipoproteins is unclear, since retinoid administration may decrease plasma HDL (5), without changing plasma apo A-I (48, 49) and apo A-II (50) concentrations in humans, whereas liver apo A-I gene expression decreases in rats in vivo (45, 51). By contrast, both in humans and

rats, retinoids induce a hypertriglyceridemia (5, 52, 53) with a mixed lipemia lipoprotein profile, such as in diabetes and familial combined hyperlipidemia (5, 35), diseases in which apo C-III is an important factor (54, 55). Since isotretinoin treatment influences neither postheparin plasma LPL activity nor plasma levels of apo C-II, the obligatory cofactor for LPL (5, 35, 56, 57), and since increased plasma apo C-III levels result in impaired peripheral lipolysis by inhibiting triglyceride-rich lipoprotein binding to the endothelial surface (18, 19), our results suggest a causal role for apo C-III in retinoid-induced dyslipidemia.

Although retinoids increase triglycerides both in humans and in rodents (5, 52, 53), important mechanistic differences may exist between species. First, the retinoid-induced hypertriglyceridemia in humans appears primarily of hepatic origin, whereas in rodents retinoids may act both by increasing hepatic VLDL synthesis, reducing hepatic VLDL uptake and decreasing peripheral lipolysis due to lowered LPL activity (52, 53). However, most studies in rodents were performed with much higher (40–50 times greater) doses than those applied in humans. Second, whereas our results clearly identify human apo C-III as a retinoid target gene, alterations in vitamin A status did not affect liver apo C-III gene expression in rodents (58). Furthermore, and in line with the absence of effects on liver apo C-III expression, VLDL particles isolated from control and isotretinoin-treated rats behave metabolically similarly (52). Finally, experiments in rats with RXR- and RAR-specific agonists suggest that the retinoid-induced hypertriglyceridemia is mediated, at least partly, by RAR (59). Although we cannot exclude that RAR may also contribute to the retinoid-induced hypertriglyceridemia in humans, our results identifying the human apo C-III as a RXR target gene indicate a role for RXR in the retinoid-induced hypertriglyceridemia in humans. Interestingly, the RAR-specific agonist Ro13-6298, the ethylester of TTNPB, does not induce hypertriglyceridemia in humans (60–62).

In conclusion, isotretinoin increases plasma apo C-III concentrations by increasing the transcriptional activity of the human apo C-III gene via RXR, but not RAR, thereby most likely contributing to the atherogenic lipoprotein profile observed after retinoid treatment.

Acknowledgments

We are grateful to S. Chopin-Delannoy, B. Derudas, and O. Vidal for expert technical assistance. B. Staels is a member of the Centre Nationale de la Recherche Scientifique.

This research was sponsored by grants from Institut Pasteur, the Région Nord-Pas de Calais, and by a "Contrat cooperation Est/Ouest" (#5REW08 INSERM).

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