Activation of the constitutive androstane receptor decreases HDL in wild-type and human apoA-I transgenic mice

David Masson,[*,†] Mohamed Qatanani,[§] Anne Laure Sberna,[*,†] Rui Xiao,[§] Jean Paul Pais de Barros,[*,†] Jacques Grober,[*,†] Valerie Deckert,[*,†] Anne Athias,[*,†] Philippe Gambert,[*,†] Laurent Lagrost,[*,†] David D. Moore,[§] and Mahfoud Assem[1,]**

Institut National de la Santé et de la Recherche Médicale U866,* Institut Federatif de Recherche 100,† Faculté de Médecine, BP 87900, 21079 Dijon Cedex, France; Department of Molecular and Cellular Biology,§ Baylor College of Medicine, Houston, TX 77030; and Clinical and Administrative Pharmacy,** College of Pharmacy, University of Iowa, Iowa City, IA 52242

Abstract **The nuclear hormone receptor constitutive androstane receptor (CAR, NR1I3) regulates detoxification of xenobiotics and endogenous molecules, and has been shown to be involved in the metabolism of hepatic bile acids and cholesterol. The goal of this study was to address potential effects of CAR on the metabolism of HDL particles, key components in the reverse transport of cholesterol to the liver. Wild-type (WT) mice, transgenic mice expressing human apolipoprotein A-I (HuAITg), and CAR-deficient ($CAR^{-/-}$) mice were treated with the specific CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP). CAR activation decreased HDL cholesterol and plasma apolipoprotein A-I (apoA-I) levels in both WT and HuAITg mice, but not $CAR^{-/-}$ mice. Both mouse apoA-I and human apoA-I were decreased by more than 40% after TCPOBOP treatment, and kinetic studies revealed that the production rate of HDL is reduced in TCPOBOP-treated WT mice. In transient transfections, TCPOBOP-activated CAR decreased the activity of the human apoA-I promoter. Although loss of CAR function did not alter HDL levels in normal chow-fed mice, HDL cholesterol, apoA-I concentration, and apoA-I mRNA levels were increased in $CAR^{-/-}$ mice relative to WT mice when both were fed a high-fat diet. We conclude that CAR activation in mice induces a pronounced decrease in circulating levels of plasma HDL, at least in part through downregulation of apoA-I gene expression.—**Masson, D., M. Qatanani, A. L. Sberna, R. Xiao, J. P. Pais de Barros, J. Grober, V. Deckert, A. Athias, P. Gambert, L. Lagrost, D. D. Moore, and M. Assem. **Activation of the constitutive androstane receptor decreases HDL in wild-type and human apoA-I transgenic mice.** ***J. Lipid Res.*** **2008.** 49: **1682–1691.**

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Constitutive androstane receptor (CAR) belongs to the nuclear receptor family. It was first described as a xenobiotic sensor that responds to a broad range of ligands and regulates the expression of genes involved in both xenobiotic and endobiotic elimination pathways (1–3). Interestingly, several studies have highlighted the role of CAR in cholesterol and bile acid metabolism (4–6) and have suggested complementary roles for CAR, pregnane X receptor (PXR), and farnesoid X receptor (FXR) in preventing bile acid toxicity. CAR may also be able to modulate plasma cholesterol transport and lipoprotein metabolism, although recent studies on the effect of CAR activators on plasma lipoprotein levels in rodents and humans are inconclusive or ambiguous. For example, treatment of epileptic patients or rats with phenobarbital, a dual CAR/PXR activator in humans (7), increases plasma cholesterol and lipoprotein levels (8, 9), and phenytoin, a nonspecific CAR activator (10–12) that activates PXR and other plasma membrane receptors and channels, increases plasma HDL concentration in humans (13, 14). On the other hand, CAR-deficient ($CAR^{-/-}$) mice exhibit higher HDL cholesterol levels than wild-type (WT) mice under cholestatic conditions following bile duct ligation, suggest-

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Abbreviations: apoA-I, apolipoprotein A-I; CAR, constitutive androstane receptor; $CAR^{-/-}$ mice, CAR-deficient mice; CE, cholesteryl ester; FCR, fractional catabolic rate; FPLC, fast-protein liquid chromatography; FXR, farnesoid X receptor; HuAITg mice, transgenic mice expressing human apolipoprotein A-I; LXR, liver X receptor; PPARα peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; WT, wild type.

[1] To whom correspondence should be addressed.
e-mail: mahfoud-assem@uiowa.edu

ing a negative effect of CAR on HDL metabolism, at least in that circumstance (6).

To specifically assess how CAR affects lipoprotein metabolism, we treated WT mice, $CAR^{-/-}$ mice (15), and transgenic mice expressing human apolipoprotein A-I (apoA-I) (HuAITg mice) (16) with the specific CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (17). Such CAR activation causes a pronounced reduction in HDL cholesterol and apoA-I levels in WT and HuAITg mice, which is absent in $CAR^{-/-}$ mice. These results demonstrate that ligand-activated CAR influences HDL metabolism in WT and HuAITg mice.

MATERIALS AND METHODS

Animals

C57Bl6 WT mice, C57Bl6 mice, $CAR^{-/-}$ mice (15), and C57Bl6 transgenic mice expressing human apoA-I under the control of its natural flanking regions (HuAITg) (16) were used in the present study. TCPOBOP (Sigma-Aldrich; St. Louis, MO) was given at the dose of 3 mg/kg to the mice (8–10 weeks old; five mice per group) by one intraperitoneal injection with 100 μl of corn oil as a vehicle. Animals were euthanized 5 days after injection. Mice had free access to water and food, and they were placed on a standard chow diet (Standard AIN-93G rodent diet containing 58.6% carbohydrate, 18.1% protein, 7.2% fat, 5.1% fiber, 3.4% ash, 10% moisture). All protocols and procedures were approved by the National Cancer Institute Division of Basic Sciences Animal Care and Use Committee and are in accordance with the National Institutes of Health guidelines.

For the high-fat diet experiment, the mice were put on a high-fat (Western) diet for 2 weeks. They were injected intraperitoneally with either 100 μl of corn oil or TCPOBOP 5 days before the end of the experiment. On the day of sample collection, the mice were fasted for 4 h (7–11 AM). The high-fat/Western diet was purchased from Harlan Tekland (Diet 88137; Harlan Tekland, Madison, WI). This diet contains 17% protein, 21% fat, 49% carbohydrate, and 0.15% cholesterol.

No differences in food intake were observed between treated and untreated mice over the 5 day period studied.

Plasma and liver tissue sampling

Animals were euthanized with isofurane, and blood samples were collected by intracardiac puncture in heparin-containing tubes that were centrifuged at 5,000 rpm for 10 min. Plasma was harvested and stored at −80°C. Livers were excised, weighed, immediately snap-frozen in liquid nitrogen, and stored at −80°C before mRNA isolation and biochemical analysis.

Plasma and HDL lipid and protein analysis

All assays were performed on a Victor2 1420 Multilabel Counter (Perkin Elmer Life Science; Boston, MA). Total cholesterol was measured by the enzymatic method using Cholesterol 100 reagent (ABX Diagnostics; Montpellier, France), and unesterified cholesterol concentration was determined by the cholesterol oxidase para-aminophenazone peroxidase method (Sigma). Esterified cholesterol concentration was calculated as the difference between total and free cholesterol. Triglycerides were determined by enzymatic methods as described previously (18). Protein concentration was measured using bicinchoninic acid reagent (Pierce Biotechnology; Rockford, IL). HDL cholesterol and non-HDL cholesterol were assayed in $d > 1.07$ and $d < 1.07$ g/ml plasma fractions, respectively.

Fractionation of plasma lipoproteins

Plasma samples (200 μl) were injected on a Superose 6 HR 10/30 column (Amersham Biosciences, Saclay, France) that was connected to a fast protein liquid chromatography (FPLC) system (Amersham Biosciences). Lipoproteins were eluted at a constant 0.3 ml/min flow rate with Tris-buffered saline containing 0.074% EDTA and 0.02% sodium azide. Total cholesterol and triglyceride concentrations were assayed in individual 0.3 ml fractions (18).

SDS-PAGE of apoB-containing lipoproteins and HDL

ApoB-containing lipoproteins and HDL were isolated by ultracentrifugation as the $d < 1.07$ g/ml fraction and the $1.07 < d < 1.21$ g/ml fraction, respectively. Densities were adjusted by the addition of KBr solutions. Samples (5 μl) were subjected to electrophoresis on 50–150 g/l polyacrylamide gradient gel (Invitrogen; Carlsbad, CA) as recommended by the manufacturer. Gels were subsequently stained by Coomassie Brilliant Blue, and scanned on a GS-800 densitometer (Bio-Rad; Hercules, CA). Molecular weights were determined by comparison with protein standards that were run in parallel with the samples.

Native polyacrylamide gradient gel electrophoresis

Total lipoproteins were separated by ultracentrifugation as the $d < 1.21$ g/ml plasma fraction with one 5.5 h, 100,000 rpm spin in a TLA100 rotor in a TLX ultracentrifuge (Beckman Instruments; Palo Alto, CA). Lipoproteins were then applied to a 15–250 g/l polyacrylamide gradient gel (Spiragel 1.5–25.0; Spiral, Couternon, France), and electrophoresis was conducted as recommended by the manufacturer. Gels were subsequently subjected to Coomassie staining, and the distribution profiles of HDL were obtained by analysis with a Bio-Rad GS-800 Imaging Densitometer. The mean apparent diameters of HDL were determined by comparison with globular protein standards (high-molecular-weight kit; Amersham Biosciences) subjected to electrophoresis together with the samples (18).

In vivo turnover studies

Mouse HDL was labeled with [^{3}H]cholesteryl ether (CE) by the following procedure: Liposomes containing phosphatidylcholine and cholesteryl-1,2-[^{3}H]hexadecylether were prepared by sonication and were incubated for 18 h at 37°C with WT HDL (1 mg of total protein) plus $d > 1.21$ infranatant from CE transfer protein-Tg mouse serum. Labeled HDL was subsequently isolated by sequential ultracentrifugation at 1.07 and 1.21 g/ml density in a TLA-100.2 rotor using a TL-100 ultracentrifuge (Beckman Instruments, Paris, France). Control and TCPOBOP-treated mice were euthanized and injected in the carotid vein with [3H]CE-labeled HDL (1×10^6 cpm). After injection, blood was taken from the tail vein at 0.08, 0.5, 1, 2, 4, 7, and 24 h for determination of radioactivity. The fractional catabolic rates (FCRs) were calculated from the decay curves of [^{3}H]CE radioactivity in plasma by fitting the data to a biexponential equation according to the method of Matthews (19). The production rates were calculated by multiplying the FCR by the plasma cholesterol pool and dividing by the body weight.

Determination of apoA-I concentration

Human apoA-I levels were determined by an immunoturbidimetric assay (Dimension Xpand Dade Behring, Paris, France). Mouse apoA-I concentration was determined by a nonimmuno-

TABLE 1. Plasma lipid parameters in WT or CAR$^{-/-}$ mice receiving or not receiving the specific CAR agonist TCPOBOP

	WT (Vehicle) (n = 5)	WT-TCPOBOP (n = 5)	CAR$^{-/-}$ (Vehicle) (n = 5)	CAR$^{-/-}$TCPOBOP (n = 5)
Total cholesterol (mmol/l)	2.01 ± 0.25	0.98 ± 0.13[a]	2.28 ± 0.13	2.38 ± 0.35[b]
HDL cholesterol (mmol/l)	1.66 ± 0.16	0.72 ± 0.06[a]	1.76 ± 0.10	1.84 ± 0.30[b]
Free cholesterol (mmol/l)	0.66 ± 0.06	0.25 ± 0.07[a]	0.59 ± 0.07	0.54 ± 0.09[b]
Esterified cholesterol (mmol/l)	1.35 ± 0.21	0.73 ± 0.11[a]	1.69 ± 0.16	1.84 ± 0.28[b]
Triglycerides (mmol/l)	0.65 ± 0.14	0.61 ± 0.11	0.58 ± 0.09	0.59 ± 0.08

CAR, constitutive androstane receptor; CAR$^{-/-}$, Car-deficient mice; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; WT, wild type. Values represent the mean ± SD.

[a] $P < 0.05$ versus vehicle (Student's *t*-test).

[b] $P < 0.05$ versus WT-TCPOBOP (Student's *t*-test).

logic method. Briefly, plasma samples (0.25 μl) were subjected to electrophoresis on 50–150 g/l polyacrylamide gradient gel (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Gels were subsequently stained by Coomassie brilliant blue and scanned on a GS-800 densitometer (Biorad, Hercules, CA). The amount of apoA-I per sample was quantified by comparison with apoA-I standards, which were run in parallel with the samples.

RNA isolation and PCR methods

Total RNA was extracted using Trizol reagent (Life Technologies; Carlsbad, CA). Specific mRNAs were analyzed by quantitative real time RT-PCR using the ABI Prism 7900HT (Applied Biosystems; Foster City, CA). Briefly, 5 μg of total RNA was reverse-transcribed into cDNA using MuMLV retrotranscriptase and oligo-dT (Life Technologies). Fifty nanograms of the cDNA mixture was used. Specific cDNAs were amplified using the following primers. Mouse apoA-I: 5′-AAGAGGATGTGGAGCTCTACC-3′ and 5′-TTCTCGCCAAGTGTCTTCAGG-3′; human apoA-I: 5′-GAAGTTGGCAGGAGGAGATGGA-3′ and 5′-TCTCGCTGAGCGTGCTCAGAT-3′; mouse Mrp4: 5′-GGTTGGAATTGTGGGCAGAA-3′ and 5′-TCGTCCGTGTGCTCATTGAA-3′. GAPDH was used as a housekeeping gene because its levels were not altered by TCPOBOP treatment and also because the amplification conditions were very close to those of apoA-I. GAPDH was amplified with primers 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCCTGTTGCTGTTGCTGTA-3′.

Values were normalized to GAPDH levels. Relative mRNA levels were evaluated using the ΔΔCt method.

Promoter reporter assay

Promoter regions −330/+67, −174/+67 and −42/+67 of the human APOA-I gene were cloned into pGL3 plasmid. HepG2 cells were maintained in DMEM supplemented with 10% FBS. One day before transfection, cells were seeded at a density of 1×10^5 cells/cm^2, and transfection was performed 24 h later with TransIT-LT1 (Mirus; Madison, WI). For each well of 24-well plates, 200 ng CAR plasmids and 100 ng pGL3 plasmids were cotransfected. Twenty four hours after transfection, cells were supplied with DMEM with 10% charcoal-stripped serum containing TCPOBOP (1 μM) or androstanol (5 μM). After 24 h incubation, luciferase activities were measured and normalized with β-galactosidase activities.

Statistical analysis

Student's *t*-test was used to determine the significance between the data means. ANOVA with Dunnett's test was used for multiple groups comparison analysis.

RESULTS

CAR activation affects HDL and apoA-I levels

Treatment with the specific CAR agonist TCPOBOP induced profound changes in plasma lipid parameters in WT mice (**Table 1**). After treatment, plasma levels of esterified cholesterol and free cholesterol were significantly decreased (~50% in all cases, $P < 0.05$), whereas

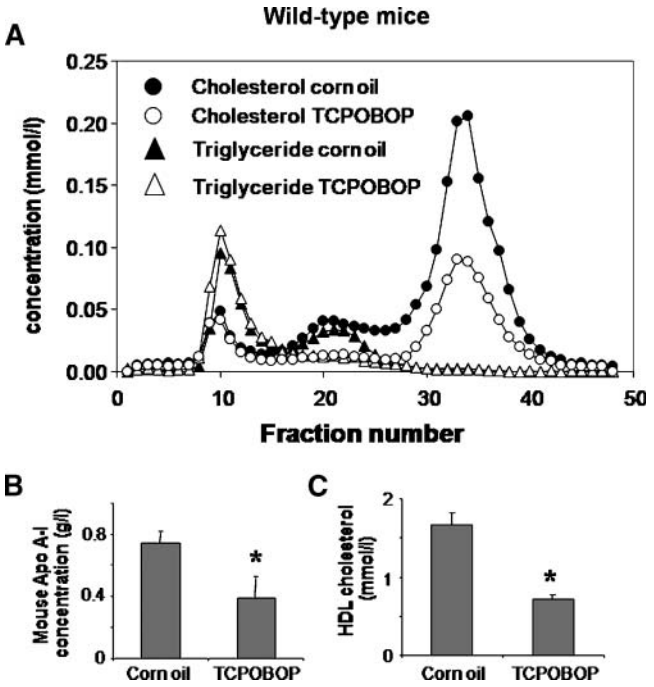

Fig. 1. Fast-protein liquid chromatography (FPLC) analysis of plasma cholesterol, plasma apolipoprotein A-I (apoA-I) concentration, and HDL cholesterol concentration in C57Bl6 wild-type (WT) mice receiving or not receiving the constitutive androstane receptor (CAR) agonist. A: Two-hundred microliters of a pool from five distinct mouse plasma samples was passed through a Superose 6 HR column, and total cholesterol and triglyceride contents of individual fractions were determined as described in Materials and Methods. B: Plasma apoA-I concentrations were determined as described in Materials and Methods. C: Plasma HDL cholesterol was determined by an enzymatic method after ultracentrifugation, as described in Materials and Methods. * Significant difference from mice receiving the control treatment ($P < 0.05$ in both cases; Student's *t*-test). Error bars indicate ± SD.

plasma triglyceride levels remained unchanged. HDL cholesterol was mostly affected by the TCPOBOP treatment in WT mice, and gel permeation chromatography analysis as well as native PAGE confirmed that plasma lipid changes were mostly explained by an alteration of the HDL fraction (**Figs. 1, 2**). A decrease in LDL levels was also observed (Figs. 1, 2), but it did not contribute to a large extent to the decrease in total plasma cholesterol levels.

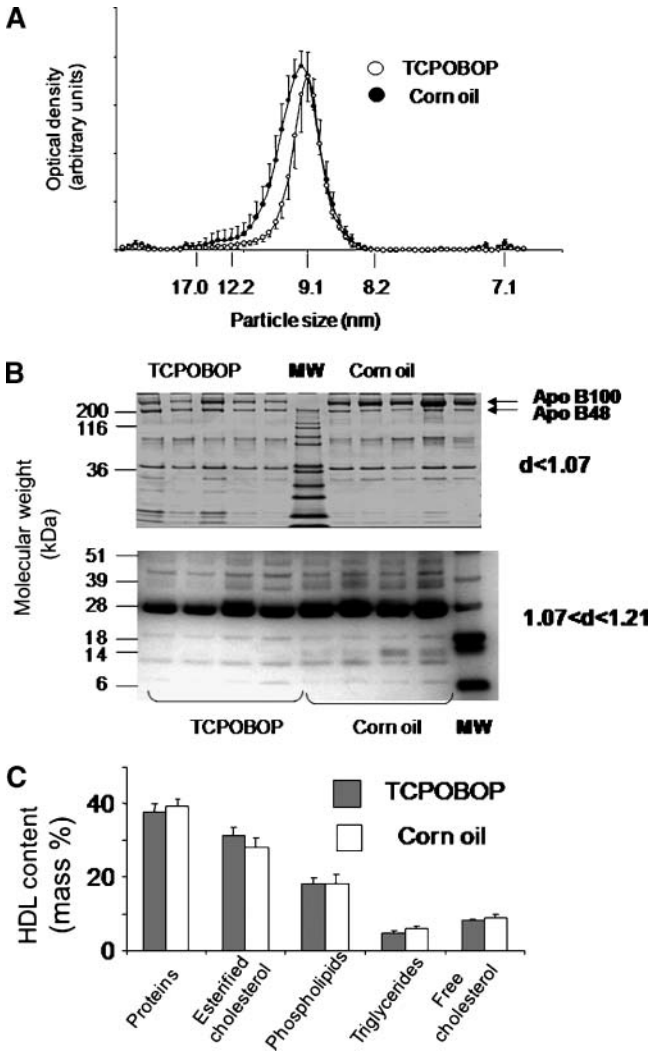

Fig. 2. Characterization of HDL from C57Bl6 WT mice receiving or not receiving the CAR agonist. A: HDL size distribution. Total plasma lipoproteins were subjected to electrophoresis on native 15–250 g/l polyacrylamide gradient gels that were stained for proteins as described in Materials and Methods. HDL profiles were obtained by image analysis, and the mean size was calculated, as compared with protein standards. B: SDS-polyacrylamide gradient gel electrophoresis of apoB-containing lipoprotein and HDL. Lipoproteins were isolated by ultracentrifugation, and apolipoproteins were separated by SDS-electrophoresis. Apolipoprotein bands were visualized after Coomassie staining, and molecular mass was calculated by comparison with the protein standards (see Materials and Methods). C: Relative composition of HDL fractions. Lipid and protein contents were determined as described in Materials and Methods. Values are expressed as mass percent of total HDL content (phospholipids + total cholesterol + triglycerides + protein).

In addition to plasma lipid changes, plasma apoA-I concentration was significantly reduced in TCPOBOP-treated WT mice (0.56 g/l in WT mice treated with TCPOBOP vs. 0.80 g/l in WT mice receiving the control vehicle, $P < 0.05$) (Fig. 1). In agreement with the tendency toward a reduction in LDL levels, a small decrease in apoB was observed by SDS-PAGE (Fig. 2).

Under basal circumstances, CAR is held in an inactive state in the hepatocyte cytoplasm. As expected, the lipoprotein phenotypes of $CAR^{-/-}$ and WT mice were similar when animals were given a chow diet, with no significant differences for any of the lipid parameters measured (Table 1), and FPLC distribution profiles of plasma lipoproteins in $CAR^{-/-}$ mice (**Fig. 3**) and WT mice (Fig. 1) were identical. The TCPOBOP-mediated changes in plasma lipids and apoA-I observed in WT mice were absent in $CAR^{-/-}$ mice. In particular, there was no reduction in HDL cholesterol or apoA-I levels after treatment of $CAR^{-/-}$ mice with TCPOBOP (Table 1, Fig. 3), and FPLC profiles were remarkably similar whether $CAR^{-/-}$ mice received or did not receive the CAR agonist (Fig. 3).

The human apoA-I transgenic mouse line (HuAITg) has been proven to be a useful model for studying in vivo the effect of molecules affecting HDL metabolism (20, 21). As

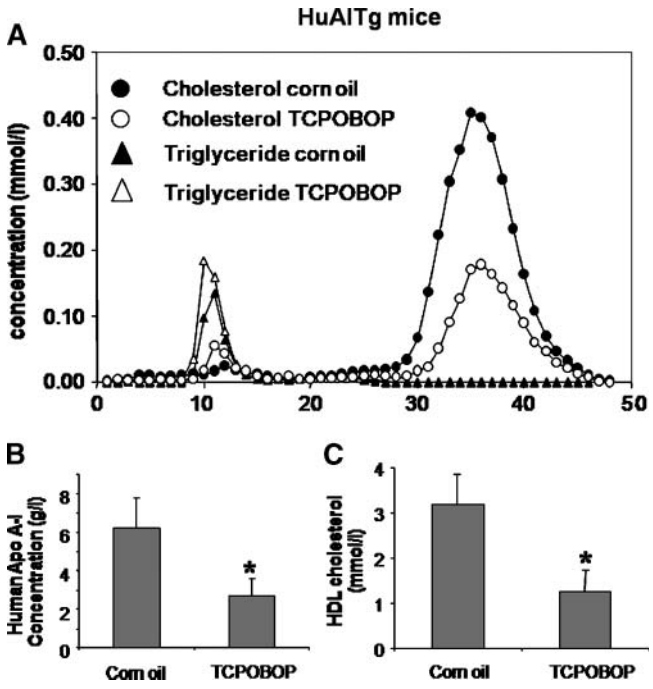

Fig. 3. FPLC analysis of plasma cholesterol, plasma apoA-I concentration, and HDL cholesterol concentration in C57Bl6 HuAITg mice receiving or not receiving the CAR agonist. A: Two-hundred microliters of a pool from five distinct mouse plasma samples was passed through a Superose 6 HR column, and total cholesterol and triglyceride contents of individual fractions were determined as described in Materials and Methods. B: Plasma apoA-I concentrations were determined as described in Material and methods. C: Plasma HDL cholesterol was determined by an enzymatic method after ultracentrifugation, as described in Materials and Methods. * Significant difference from mice receiving the control treatment ($P < 0.05$ in both cases; Student's *t*-test). Error bars indicate ± SD.

previously reported (16), HuAITg mice showed increased basal apoA-I and total cholesterol levels due to an increase in the HDL fraction (**Fig. 4**, and Table 1, and **Table 2**) (16, 21). Again, and as observed above with WT mice, TCPOBOP administration produced profound changes in the plasma lipoprotein profile of HuAITg mice, with ~60% reductions in total cholesterol, esterified cholesterol, HDL cholesterol, and human apoA-I levels ($P < 0.05$ in all cases) (Fig. 4). As observed in WT mice, the TCPOBOP treatment had no effect on the plasma triglyceride level in HuAITg mice (Table 2).

TABLE 2. Plasma lipid concentration in HuAITg mice receiving or not receiving the specific CAR agonist TCPOBOP

	HuAITg (Vehicle) (n = 5)	HuAITg TCPOBOP (n = 5)
Total cholesterol (mmol/l)	3.83 ± 0.56	1.54 ± 0.55[a]
HDL cholesterol (mmol/l)	3.18 ± 0.69	1.26 ± 0.48[a]
Free cholesterol (mmol/l)	0.79 ± 0.08	0.35 ± 0.08[a]
Esterified cholesterol (mmol/l)	3.04 ± 0.50	1.19 ± 0.48[a]
Triglycerides (mmol/l)	0.42 ± 0.06	0.58 ± 0.15

HuAITg mice, transgenic mice expressing human apolipoprotein A-I.
[a] $P < 0.05$ versus vehicle (Student's *t*-test).

TCPOBOP treatment reduces the production of HDL

To investigate the effect of CAR activation on HDL kinetics, HDL was labeled with [^{3}H]CE. This analog of CE behaves in plasma as CE but cannot be hydrolyzed in tissues. The catabolism of CE-labeled HDL tended to be slightly increased in TCPOBOP-treated mice (**Fig. 5**), but the FCRs were not statistically different between control and TCPOBOP-treated mice (3.82 ± 0.41 vs. 4.58 ± 0.75 pools/day, respectively; not significant). Conversely, the calculated production rate was significantly reduced in the TCPOBOP group as compared with the control group (78.9 ± 15.0 vs. 97.2 ± 5.29 mg/day^{-1}/g^{-1}; $P < 0.05$).

CAR activation is associated with the repression of hepatic apoA-I expression

As expected, the expression of Cyp2b10 (not shown) and MRP4, typical positive CAR target genes (22), was markedly induced by TCPOBOP in the liver of WT and HuAITg mice, whereas no significant changes were observed in CAR$^{-/-}$ mice (**Fig. 6**). In contrast, TCPOBOP treatment of WT mice produced a 30% reduction in the liver mRNA levels of apoA-I ($P < 0.05$; Student's *t*-test). In HuAITg mice, the TCPOBOP treatment produced a 3-fold reduction in the hepatic level of mouse apoA-I mRNA, and a 5-fold reduction in the hepatic level of

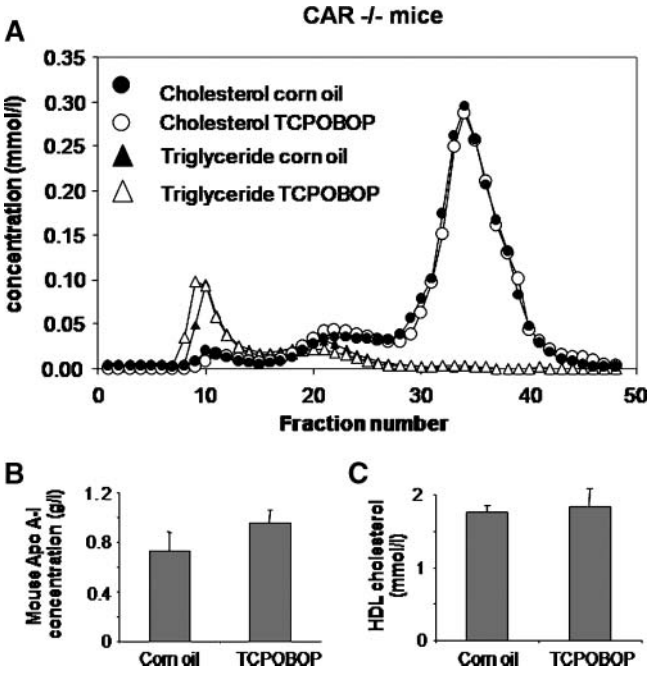

Fig. 4. FPLC analysis of plasma cholesterol, plasma apoA-I concentration, and HDL cholesterol concentration in C57Bl6 CAR-deficient (CAR$^{-/-}$) mice receiving or not receiving the CAR agonist. A: Two-hundred microliters of a pool from five distinct mouse plasma samples was passed through a Superose 6 HR column, and total cholesterol and triglyceride contents of individual fractions were determined as described in Materials and Methods. B: Plasma apoA-I concentrations were determined as described in Material and Methods. C: Plasma HDL cholesterol was determined by an enzymatic method after ultracentrifugation, as described in Materiala and Methods. * Significant difference from mice receiving the control treatment ($P < 0.05$ in both cases; Student's *t*-test). Error bars indicate ± SD.

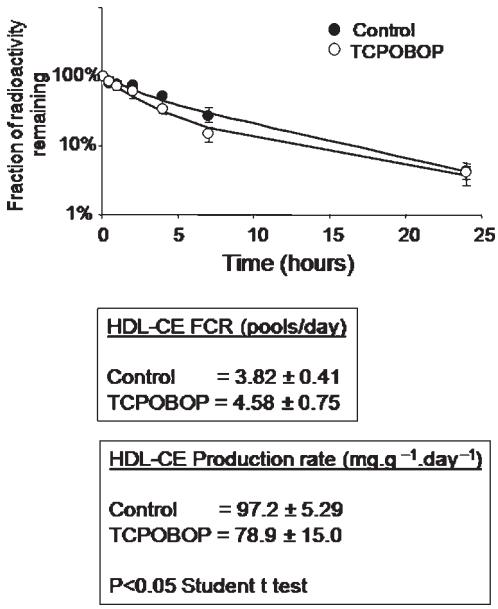

Fig. 5. Plasma kinetics of HDL labeled with [3H]cholesteryl hexadecyl ether in control or TCPOBOP-treated WT mice. The tracer was administered by jugular vein injection, and blood samples were taken at the tail vein at the indicated time points and analyzed for radioactivity on a scintillation counter. Values are the fraction of the injected dose remaining at each time point. The curves were fitted using a biexponential equation. Fractional catabolic rate and production rate values were calculated as described in Materials and Methods. Data are given as mean ± SD; n = 4 mice/group for each point.

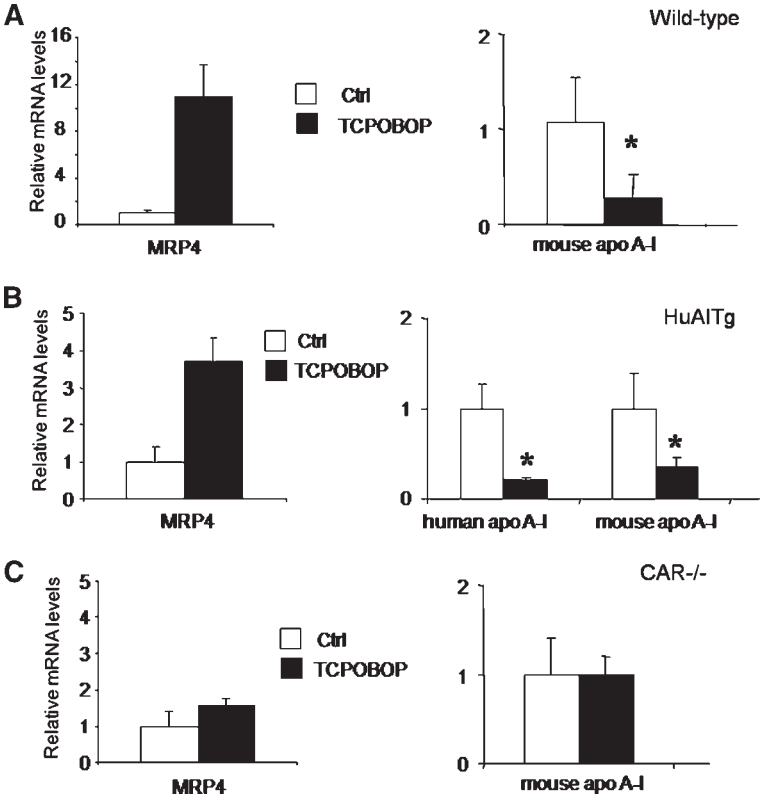

Fig. 6. ApoA-I and MRP4 mRNA levels in the liver of C57Bl6 WT mice (A), HuAITg mice (B), and CAR$^{-/-}$ mice (C) treated or not treated with the CAR agonist TCPOBOP. Total RNA was extracted from the liver, and apoA-I and MRP4 mRNA levels were determined by real-time RT-PCR as described in Materials and Methods. Data were standardized with GAPDH as standard, and mRNA levels in mice treated with corn oil were set at 1.00. Values are means ± SD. * Significantly different from untreated group ($P < 0.05$; Student's *t*-test).

human apoA-I ($P < 0.05$ in both cases; Student's *t*-test). Again, observed changes were shown to relate specifically to CAR activation, because no significant differences in apoA-I gene expression were observed in CAR$^{-/-}$ mice treated with TCPOBOP.

CAR represses the activity of the human apoA-I promoter

To test the hypothesis that CAR downregulates APOA-I at the transcriptional level, three different 5′ deletion constructs of the human APOA-I promoter were cotransfected in HepG2 cells, together with an expression vector for CAR, a mutant version of CAR lacking AF-2 function or with the empty vector as control (**Fig. 7**). Cells were treated with DMSO, TCPOBOP, or the inverse CAR agonist androstanol. CAR markedly repressed the activity of the −330 bp apoA-I promoter under basal conditions, and this effect was both enhanced by TCPOBOP and partially reversed by the inverse agonist androstanol. In all cases, the effects were less pronounced with CAR lacking AF-2 function. The repression was still observed with the −174 bp apoA-I promoter construct, but was abolished for the −41 bp construct. Taken together, these results indicate that the region −174/−43 of the apoA-I promoter contains a *cis*-acting element negatively regulated by CAR. Interestingly, this region is highly conserved and is more than 90% homologous to the mouse region (Fig. 7B).

CAR$^{-/-}$ mice display a distinct lipoprotein phenotype when fed a high-fat diet

WT and CAR$^{-/-}$ mice were fed a high-fat diet for 2 weeks prior to TCPOBOP treatment. As with the chow-fed mice, CAR activation decreased cholesterol levels in the WT but not the CAR$^{-/-}$ mice. Under these conditions, CAR$^{-/-}$ mice display modestly elevated total cholesterol, HDL cholesterol, and apoA-I levels relative to the WT mice (**Table 3**, **Fig. 8**). Similar observations were made with apoA-I mRNA levels, with significantly higher levels in CAR$^{-/-}$ relative to WT mice (**Fig. 9**).

DISCUSSION

CAR, a member of the nuclear receptor family was initially described as a xenobiotic sensor (1–3). More recently, its key role in elimination pathways of potentially harmful endogenous molecules has been recognized. In particular, a number of studies have pointed out the relationship between CAR and hepatic cholesterol/bile acid metabolism (4–6, 23). Specifically, CAR is able to protect the liver against bile acid toxicity by inducing expression of genes encoding proteins involved in *1*) the synthesis of hydrophilic 6-OH bile acids at the expense of more-hydrophobic and toxic species (24, 25), *2*) the sulfonation of bile acids (23, 26), and *3*) the excretion of conjugated bile acids (23).

The present study demonstrates that activation of CAR by TCPOBOP decreases plasma HDL levels in WT mice but not in CAR$^{-/-}$ mice. CAR is inactive under basal conditions and, as expected, CAR deficiency did not result in altered plasma lipoprotein profiles of chow-fed animals. After feeding a high-fat diet for 2 weeks, however, CAR$^{-/-}$ mice displayed a modest increase in cholesterol

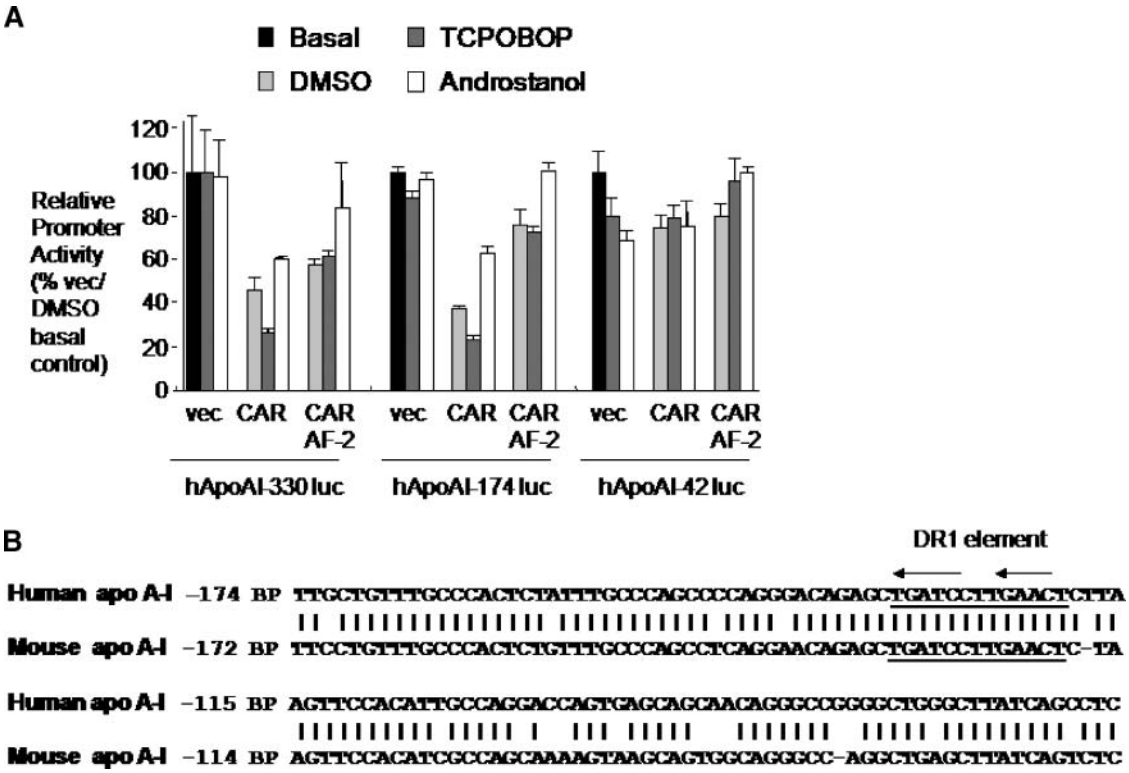

Fig. 7. A: Effect of CAR on human apoA-I gene promoter activity. The indicated human apoA-I promoter/luciferase constructs were cotransfected with expression vectors for CAR, a mutant version lacking AF-2 function, or the vector alone, along with a cytomegalovirus-β-galactosidase internal control. Transfected cells were incubated with either the DMSO vehicle, the CAR agonist TCPOBOP, or the inverse agonist androstanol. Due to the decrease in basal expression with shorter promoter fragments, β-galactosidase-normalized expression for each promoter construct is shown relative to the vector alone plus DMSO basal control (100%). CAR did not repress expression from a control thymidine kinase-luciferase reporter. B: Sequence alignment of mouse and human apoA-I promoters.

levels relative to WT mice. This is consistent with an increase in plasma HDL observed in $CAR^{-/-}$ mice placed under cholestatic conditions (6). These observations support the concept of a negative effect of activated CAR on HDL levels in mice. Similarly, the liver X receptors (LXRs) showed no major effect on HDL profile in animals fed standard chow, whereas a major regulatory function of LXR in HDL metabolism was found in animals treated with either oxidized cholesterol or other specific LXR agonists (27, 28).

To determine the mechanisms that account for the reduced HDL/apoA-I levels in TCPOBOP-treated animals, kinetic analysis of HDL labeled with [^{3}H]CE was performed. The production rate of HDL was significantly lower in the TCPOBOP-treated group, indicating, in accordance with reduced apoA-I mRNA levels, that an alteration of the synthesis of HDL contributed at least in part to the observed phenotype.

ApoA-I is a major structural component of HDL (16), and its expression level is a leading determinant of circulating HDL levels. Interestingly, in HuAITg mice, both mouse and human apoA-I mRNA levels were markedly decreased by TCPOBOP treatment, and substantial reductions in plasma levels of human apoA-I were observed. This concordance in the alterations of both mouse and human apoA-I gene expression contrasts with opposite

TABLE 3. Plasma lipid parameters in WT and $CAR^{-/-}$ mice on high-fat diet receiving or not receiving the specific CAR agonist TCPOBOP

	Wild-Type High-Fat (n = 4)	Wild-Type High-Fat TCPOBOP (n = 5)	$CAR^{-/-}$ High-Fat (n = 5)	$CAR^{-/-}$ High-Fat TCPOBOP (n = 5)
Total cholesterol (mmol/l)	4.43 ± 0.29	2.96 ± 0.33[a]	5.65 ± 0.55[a]	5.90 ± 0.98[b]
HDL cholesterol (mmol/l)	2.95 ± 0.24	2.19 ± 0.16[a]	4.06 ± 0.38[a]	3.65 ± 1.04[b]
Free cholesterol (mmol/l)	1.18 ± 0.27	0.81 ± 0.18	1.14 ± 0.11	1.41 ± 0.32[b]
Esterified cholesterol (mmol/l)	3.25 ± 0.05	2.15 ± 0.24[a]	4.51 ± 0.58[a]	4.50 ± 0.72[b]
Triglycerides (mmol/l)	1.45 ± 0.47	2.84 ± 0.72[a]	1.34 ± 0.17	1.15 ± 0.2[b]

Values represent the mean ± SD.
[a] $P < 0.05$ versus wild-type high-fat (Student's *t*-test).
[b] $P < 0.05$ versus wild-type high-fat TCPOBOP (Student's *t*-test).

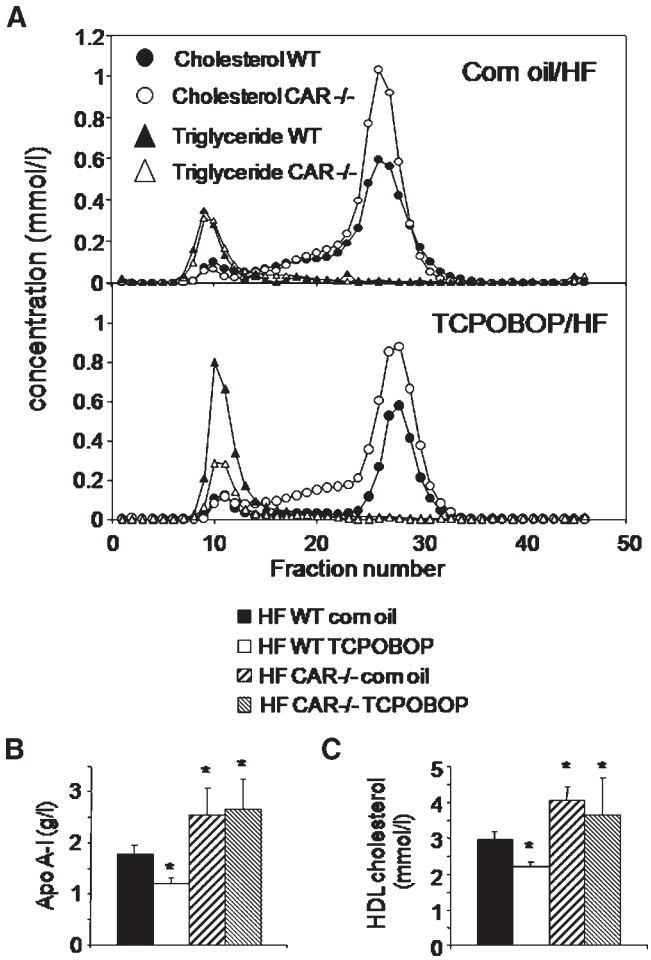

Fig. 8. FPLC analysis of plasma cholesterol, plasma apoA-I concentration, and HDL cholesterol concentration in C57Bl6 WT and $CAR^{-/-}$ mice fed a high-fat diet and receiving or not receiving the CAR agonist. A: Two-hundred microliters of a pool from five distinct mouse plasma samples was passed through a Superose 6 HR column, and total cholesterol and triglyceride contents of individual fractions were determined as described in Materials and Methods. B: Plasma apoA-I concentrations were determined as described in Materials and Methods. C: Plasma HDL cholesterol was determined by an enzymatic method after ultracentrifugation as described in Materials and Methods. * Significant difference with WT mice receiving the control treatment ($P < 0.05$ in both cases; ANOVA and Dunnett's test). Error bars indicate ± SD.

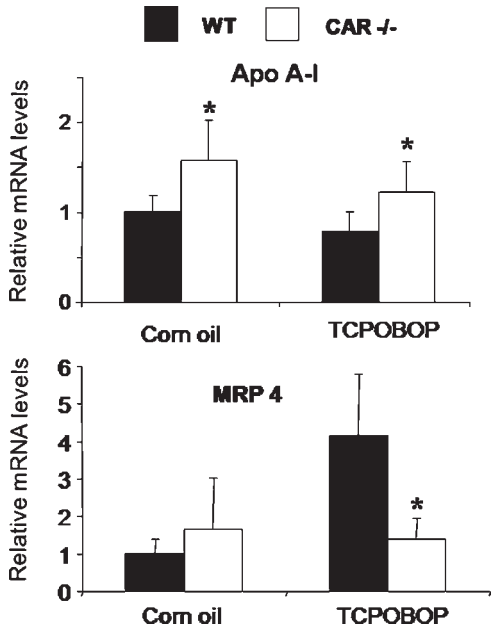

Fig. 9. ApoA-I and MRP4 mRNA levels in the liver of C57Bl6 WT mice and $CAR^{-/-}$ mice fed a high-fat diet and treated or not treated with the CAR agonist TCPOBOP. Total RNAs were extracted from the liver, and apoA-I and MRP4 mRNA levels were determined by real-time RT-PCR as described in Materials and Methods. Data were standardized with GAPDH as standard, and mRNA levels in mice treated with corn oil were set at 1.00. Values are means ± SD. * Significant difference with WT mice ($P < 0.05$; Student's *t*-test).

tendencies reported in some earlier studies of the transcriptional regulation of the apoA-I gene. In particular, peroxisome proliferator-activated receptor α (PPARα) agonists are known to transactivate the human apoA-I gene, whereas they repress mouse apoA-I gene expression (21). The present study indicates that this important limitation of the mouse model does not apply to the regulation of apoA-I expression by CAR agonists that seem to produce similar effects on rodent and human apoA-I. However, observations concerning the effect of CAR on plasma lipoprotein metabolism in humans still remain unclear. Human studies were conducted with either phenobarbital or phenytoin, which, unlike TCPOBOP, are nonspecific CAR agonists inducing only moderate changes in plasma HDL cholesterol and apoA-I levels (8, 10, 11, 13). Furthermore, these drugs are known to also activate PXR and other metabolic pathways as well. This nonspecific action may explain some phenotypic variation. We used only TCPOBOP as CAR activator, because it is the only specific mouse CAR agonist available so far. Phenobarbital is a dual PXR/CAR agonist; therefore, additional studies are clearly needed to analyze its effects on lipoprotein metabolism in mice lacking the nuclear receptor PXR.

In the present study, we demonstrate that CAR acts at the transcriptional level by inhibiting the activity of the human apoA-I promoter. The detailed mechanism of this repression is uncertain, but several hypotheses are plausible. First, CAR might exert a direct effect on the apoA-I gene promoter. This hypothesis is supported by the fact that CAR behaves as either an activating factor or a repressing factor with several genes, such as carnitine palmitoyl transferase or phosphoenolpyruvate carboxykinase, that are negatively regulated by phenobarbital in a CAR-dependent manner (29). Alternatively, CAR might act indirectly, by interfering with other transcriptional regulatory pathways involved in apoA-I gene expression. This might involve a direct competition at the gene promoter level, as reported for LXR (30) or for the recruitment of common coactivators such as PPARγ, proliferator-activated receptor γ coactivator-1α (Pgc-1α), and the glucocorticoid receptor-interacting protein 1 (Grip-1), as reported for FXR and PXR and recently for CAR and HNF-4 (31–33).

Interestingly, the −174/−41 region, which is required for the negative effects of CAR, includes sites B and C of the apoA-I liver-specific enhancer (34). As stated earlier, this region of the apoA-I promoter shows more than 90% homology between humans and mice, suggesting similar mechanisms of regulation in the two species. Although the region contains no evident DR4 elements, which are the preferred binding sites for CAR, it includes a well-characterized DR1 element (−133 to −120) that is crucial for apoA-I promoter activity (Fig. 7B). This DR1 has been described previously and is able to bind a large variety of homodimers or heterodimers of nuclear receptors (LR-HI, FXR, HNF4, RXR) (35, 36, 37). This further suggests that CAR activation may interfere with other transcriptional pathways (34–36).

In conclusion, the present study demonstrates that CAR activation alters plasma HDL metabolism in WT and HuAITg mice. It is possible that inhibiting the HDL-mediated reverse cholesterol transport pathway contributes to the ability of CAR agonists to preserve normal liver function via downregulation of bile acid synthesis and toxicity.

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