Repression of hepatocyte nuclear factor 4 alpha by AP-1 underlies dyslipidemia associated with retinoic acid[S]

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Abstract ***All-trans*** **retinoic acid (atRA) is used to treat certain cancers and dermatologic diseases. A common adverse effect of atRA is hypercholesterolemia; cytochrome P450 (CYP) 7A repression is suggested as a driver. However, the underlying molecular mechanisms remain unclear. We investigated CYP7A1 expression in the presence of atRA in human hepatocytes and hepatic cell lines. In HepaRG cells, atRA increased cholesterol levels dose-dependently alongside dramatic decreases in CYP7A1 expression. Lentiviral-mediated CYP7A1 overexpression reversed atRA-induced cholesterol accumulation, suggesting that CYP7A1 repression mediated cholesterol accumulation. In CYP7A1 promoter reporter assays and gene-knockdown studies, altered binding of hepatocyte nuclear factor 4 α (HNF4α) to the proximal promoter was essential for atRA-mediated CYP7A1 repression. Pharmacologic inhibition of c-Jun N-terminal kinase (JNK) and ERK pathways attenuated atRA-mediated CYP7A1 repression and cholesterol accumulation. Overexpression of AP-1 (c-Jun/c-Fos), a downstream target of JNK and ERK, repressed CYP7A1 expression. In DNA pull-down and chromatin immunoprecipitation assays, AP-1 exhibited sequence-specific binding to the proximal CYP7A1 promoter region overlapping the HNF4α binding site, and atRA increased AP-1 but decreased HNF4α recruitment to the promoter. Collectively, these results indicate that atRA activates JNK and ERK pathways and the downstream target AP-1 represses HNF4α transactivation of the CYP7A1 promoter, potentially responsible for hypercholesterolemia.—** Won, K-J., J-S. Park, and H. Jeong. **Repression of hepatocyte nuclear factor 4 alpha by AP-1 underlies dyslipidemia associated with retinoic acid.** ***J. Lipid Res.*** **2019.** 60: **794–804.**

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Retinoids (vitamin A and its derivatives) modulate physiological processes including proliferation, differentiation, and apoptosis, via regulation of multiple genes (1, 2). These regulatory effects of retinoids are mediated mainly by retinoic acids (RAs), the bioactive metabolites of retinoids. *All-trans* RA (atRA) is the most biologically active RA and is used effectively for the treatment of cancers and dermatological disorders (3–6). Of note, one of the most prevalent side effects of retinoid drug therapy is hypercholesterolemia (affecting 31% of patients) that potentially promotes atherosclerosis (6–8).

Cholesterol homeostasis is regulated mainly by the rates of cholesterol synthesis and elimination in the liver. These processes are modulated by the levels of the enzymes catalyzing the rate-limiting steps: the conversion of HMG-CoA to mevalonate by HMG-CoA reductase (HMGCR) for cholesterol synthesis (9) and cytochrome P450 7A1 (CYP7A1)-mediated conversion of cholesterol to bile acids for elimination (10). Previous studies reported that atRA represses *CYP7A1* expression in HepG2 cells and human hepatocytes, as well as in mouse liver (11, 12), but the detailed molecular mechanism of how atRA leads to *CYP7A1* repression is unclear. Furthermore, the effects of retinoids on HMGCR expression/activity in the liver remain unknown.

The expression of *CYP7A1* is tightly controlled at transcriptional and posttranscriptional levels (13–18). Multiple microRNAs are known to decrease mRNA stability by targeting sequences in the 3′-untranslated region of *CYP7A1* mRNA (19). The promoter of *CYP7A1* contains conserved response elements for multiple transcription factors with different functionality (20). For example, pregnane X receptor (PXR) represses the *CYP7A1* promoter (21, 22), whereas hepatocyte nuclear factor 4α (HNF4α) and liver

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Abbreviations: atRA, *all-trans* retinoic acid; ChIP, chromatin immunoprecipitation; CST, Cell Signaling Technology; CYP7A1, cytochrome P450 7A1; FXR, farnesoid X receptor; HMGCR, HMG-CoA reductase; HNF4α, hepatocyte nuclear factor 4α; JNK, c-Jun N-terminal kinase; LRH-1, liver receptor homolog-1; PXR, pregnane X receptor; qPCR, quantitative PCR; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid receptor response element; ROS, reactive oxygen species; RXR, retinoid X receptor; SHP, small heterodimer partner.

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receptor homolog-1 (LRH-1) activates the promoter (13, 23–25).

HNF4α is functionally modulated by multiple mechanisms, including intracellular signaling and protein-protein interactions. For example, activation of MAPKs, such as c-Jun N-terminal kinases (JNKs), ERKs, and p38, can inhibit HNF4α activity (23, 26, 27). The AP-1 protein family members serve as downstream effectors of ERK and JNK signaling pathways. Upon activation, these proteins form homodimers or heterodimers to regulate the expression of their target genes. Bile acids are known to activate c-Jun, a member of the AP-1 protein family, that interacts with HNF4α, leading to *CYP7A1* repression (28). Additionally, transcriptional activity of HNF4α can be inhibited by its interaction with corepressors, such as small heterodimer partner (SHP) (29–31). Of note, atRA is known to induce *SHP* expression in human hepatocytes (11) and activate MAPK in multiple tissues, including breast cancer and intestinal cells (32, 33).

Retinoids can regulate gene transcription by binding to their cognate receptors, RA receptors (RARs) and retinoid X receptors (RXRs). The complex subsequently binds to the RAR response element (RARE), two direct repeats of hexameric sequences (AGGTCA-like) with 5 base pair spacers (i.e., DR5), and modulates the promoter activities of target genes (34, 35). Functional RARE was previously identified in the *Cyp7a1* promoter of rodents (13), but it is unknown whether the respective sequences in the human *CYP7A1* promoter are functional. RXR binding of retinoids can also lead to activation of its permissive binding partners, including PXR (36) and farnesoid X receptor (FXR). FXR transactivates the *SHP* promoter, and SHP, in turn, can repress HNF4α transactivation of the *CYP7A1* promoter (24, 25, 37).

In this study, we report that atRA increases cholesterol levels, potentially by reducing *CYP7A1* expression. We found that atRA activates the MAPK/AP-1 signaling pathway and inhibits the recruitment of HNF4α to the *CYP7A1* promoter, leading to *CYP7A1* repression. Results from transient transfection and promoter reporter assays also suggest that the involvement of multiple nuclear receptors (i.e., PXR, FXR, LRH-1, RAR, RXR, and SHP) in the atRA action on *CYP7A1* expression is unlikely.

MATERIALS AND METHODS

Cell culture

HepaRG cells were purchased from Biopredic International (Saint Grégoire, France) and cultured in Williams' medium E supplemented with 10% FBS (Gemini, West Sacramento, CA), 5 μg/ml insulin, 2 mM L-glutamine, and 50 μM hydrocortisone hemisuccinate for 2 weeks. Confluent HepaRG cells were cultured in the same medium containing 2% DMSO (differentiation medium) for another 2 weeks. Medium was replaced every 2–3 days. Fully differentiated HepaRG cells were used as a liver model.

Primary human hepatocytes were obtained from the Liver Tissue Cell Distribution System (Pittsburgh, PA; funded by National Institutes of Health Contract HHSN276201200017C). Upon receipt, medium was replaced with Williams' medium E supplemented with 0.1 μM dexamethasone, 2 mM L-glutamine, and 1% ITS solution (catalog no. I3146, Sigma). After stabilization for 24 h, cells were used for experiments.

HEK293T and HepG2 cells were obtained from ATCC (Manassas, VA) and cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. All cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

atRA treatment

A stock solution of atRA (catalog no. PHR1187, Sigma) was prepared at a concentration of 10 mM in DMSO. Cells were starved in FBS-free medium overnight before atRA treatment. The final DMSO concentrations in medium were 2.01% or 0.01% (2.01% for differentiated HepaRG cells, 0.01% for human hepatocytes and HepG2 cells). The media containing atRA were replaced every 24 h.

Total cholesterol quantification

Total cholesterol levels were determined using the cholesterol assay kit (Cell Biolabs, San Diego, CA), according to the manufacturer's instructions. Briefly, cells were washed with ice-cold PBS and homogenized in a mixture of chloroform, isopropanol, and NP-40 (7:11:0.1). After centrifugation at 15,000 *g*, organic phase was transferred to a new tube and dried until the solvents were removed. The dried pellets were dissolved in the assay diluent that contains cholesterol esterase and incubated with the cholesterol reaction reagent for 45 min at 37°C. Absorbance was measured at 540 nm.

Total RNA extraction and qPCR

Total RNA was extracted using Trizol reagent (Thermo Fisher) and was reverse-transcribed into cDNA using a High-Capacity cDNA reverse-transcription kit (Thermo Fisher). Quantitative PCR (qPCR) was performed using the StepOnePlus™ real-time PCR system with PrimeTime probes (Integrated DNA Technologies, Coralville, IA). The following probes were used: *CYP7A1* (Hs.PT.58.21408221), *HMGCR* (Hs.PT.58.41105492), *HNF4A* (Hs.PT.58.22303533), *SHP* (Hs.PT.58.38586840), *ABCA1* (Hs.PT.58.27452429), *ABCG5* (Hs.PT.58.40909601), *ABCG8* (Hs.PT.58.40210561), and *GAPDH* (Hs.PT.39a.22214836). The values were expressed as mRNA levels normalized to those of *GAPDH* ($2^{-\Delta\Delta ct}$ method).

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mM EDTA) containing protease/phosphatase inhibitor cocktails (Roche, Mannheim, Germany). Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated with 5% skim milk in TBS containing 0.1% Tween 20 (TBST) for 1 h and incubated with primary Abs against CYP7A1 (catalog no. ab79847, Abcam), HNF4α (catalog no. PP-H1415-00, R&D Systems), SHP (catalog no. sc-271511, Santa Cruz), p-P38 [catalog no. 4511, Cell Signaling Technology (CST)], P38 (catalog no. 8690, CST), p-JNK (catalog no. 4668, CST), JNK (catalog no. 9252, CST), p-ERK (catalog no. 4370, CST), ERK (catalog no. 4695, CST), p-MAPKAPK-2 (p-MK2; catalog no. 3007, CST), MAPKAPK-2 (MK2; catalog no. 3042, CST), p-c-Jun (catalog no. 2361, CST), c-Jun (catalog no. 9165, CST), c-Fos (catalog no. 2250, CST), and RARα (catalog no. sc-773, Santa Cruz) at 4°C overnight. Membranes were washed with TBST and incubated with secondary Ab for 1 h. Proteins were visualized with chemiluminescent substrate (Thermo Fisher).

Chromatin immunoprecipitation assay

Cells were fixed in medium containing 1% paraformaldehyde at room temperature for 10 min and incubated in 125 mM glycine solution for 5 min to quench cross-links. Cells were washed with

PBS and lysed in hypotonic buffer solution (20 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP-40, and protease and phosphatase inhibitor cocktails; Roche) for 10 min. After centrifugation at 10,000 *g* at 4°C for 1 min, supernatants were discarded, and nuclear pellets were rinsed with PBS and sonicated in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, and protease and phosphatase inhibitor cocktails) to shear DNA to the length ranging from 200 to 1,000 bp. After centrifugation at 16,000 *g* at 4°C for 10 min, supernatants were incubated with Ab-conjugated Dynabeads (Thermo Fisher) overnight. Chromatin immunoprecipitation (ChIP)-grade Abs against HNF4α (catalog no. PP-H1415-00, R&D Systems), c-Jun (catalog no. 9165, CST), and c-Fos (catalog no. 2250, CST) were conjugated to Dynabeads. The beads were washed with low-salt buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA), high-salt buffer (20 mM Tris pH 8.0, 500 mM NaCl, 1% Triton X-100, and 1 mM EDTA), and TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). Immune complexes were eluted in elution buffer (1% SDS and 0.1 M NaHCO3), and cross-linking was reversed by adding NaCl and heating at 65°C, followed by protease K treatment. DNA fragments were recovered using a Qiaquick PCR kit (Qiagen) and quantified by qPCR. The following PrimeTime probes were used to detect *CYP7A1* promoter region: 5′-ggtctctgattgctttggaac-3′ (forward), 5′-catagtatccagatccattaacttgag-3′ (reverse), and 5′-acctgtggacttagttcaaggcca-3′ (probe). The signal was normalized by that from the amplified fragment without immunoprecipitation and expressed as percent input.

Nuclear extraction

Cells were washed with PBS and lysed in cytoplasmic extract buffer (20 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP-40, and protease/phosphatase inhibitor cocktails) for 10 min. The cytosolic fraction was isolated by centrifugation at 10,000 *g* at 4°C for 1 min. The pellets were rinsed with ice-cold PBS and lysed in nuclear extract buffer (20 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, and protease/phosphatase inhibitor cocktails) at 4°C for 1 h. The nuclear fraction was isolated by centrifugation at 16,000 *g* at 4°C for 10 min. The supernatants were used in the DNA pull-down assay.

DNA pull-down assay

The 5′ biotin-labeled oligonucleotides and nonlabeled oligonucleotides (competitor) were synthesized by Integrated DNA Technologies. The oligonucleotide sequences were as follows: WT, 5′-cctgtggacttagttcaaggccagttactacc-3′; m1, cctgccacattagttcaaggccagttactacc-3′; and m2, 5′-cctgtggacttctcccaaggccagttactacc-3′. Duplex oligonucleotides (4 μg)-conjugated Dynabeads (M-280 Streptavidin, Thermo Fisher) were incubated with nuclear extracts (200 μg) at room temperature for 2 h. The beads were washed with PBS, and proteins were eluted by boiling in 1× sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.2% bromophenol blue). The proteins were separated by SDS-PAGE followed by Western blot analysis.

Plasmids

The insert DNA of the *CYP7A1* promoter between −200 and +50 was synthesized by Genscript (Piscataway, NJ) and cloned into pGL3 vector to generate pGL3-CYP7A1 (−200/+50) plasmid. Deletion fragments of the *CYP7A1* promoter were PCR-amplified using pGL3-CYP7A1 (−200/+50) as a template and cloned into pGL3 vector to generate pGL3-CYP7A1 (−100/+50 and −50/+50) plasmids. The mutations in pGL3-CYP7A1 constructs were made by Genscript. Expression vectors for c-Jun and c-Fos were purchased from Genscript. pcDNA3-HNF4A plasmid was received from Frances M. Sladek (University of California, Riverside, CA).

Luciferase reporter assay

HepG2 cells were transfected with promoter reporter constructs with or without expression vectors using FuGENE HD reagent (Promega) for 24 h and treated with atRA for 24 h. Luciferase activity was measured using GloMax luminometer (Promega) and normalized to the Renilla luciferase activity.

Lentiviral infection

Flag-tagged *CYP7A1* clone was purchased from Genscript. Coding region of Flag-*CYP7A1* was PCR-amplified and cloned into VVPW lentiviral expression vector (gift of G. L. Gusella, Mount Sinai Hospital, New York) to create VVPW-Flag-CYP7A1 plasmid. HEK293T cells were cotransfected with VVPW-Flag-CYP7A1, pCMV-VSVG, and psPAX2 plasmids (38, 39) in a ratio of 3:2:1 using FuGENE HD reagent according to the manufacturer's instructions to produce virus harboring Flag-CYP7A1. Control virus was produced using empty VVPW vector together with pCMV-VSVG and psPAX2. After 12 h transfection, medium was replaced, and viruses were produced for 48 h. Medium containing virus particles was filtered through a 0.45 μm filter, concentrated by Lenti-X Concentrator (Clontech). The viral particles were titrated by a Lenti-X titration kit (Takara). HepaRG cells and human hepatocytes were transduced with equal amounts of lentiviral particles in the presence of polybrene (2 μg/ml).

Gene knockdown

Gene expression was silenced by siRNA transfection using Dharmafect 1 (Dharmacon, Lafayette, CO), according to the manufacturer's instruction. The following siRNAs (Dharmacon) were used: control (catalog no. D-001206-14), *HNF4A* (catalog no. M-003406-02), and *SHP* (catalog no. M-003410-01).

Statistical analysis

All values were presented as means ± SD. For comparison of two groups, statistical differences were determined by Student's *t*-test. For statistical testing of multiple groups, one-way ANOVA followed by posthoc Tukey's test was performed.

RESULTS

atRA treatment increases cholesterol levels through *CYP7A1* repression

We first examined whether atRA affects total cholesterol level in human liver cells. Primary hepatocytes and differentiated HepaRG cells have been used to recapitulate the processes of the intact liver (40, 41) and thus were chosen as model systems. atRA increased total cholesterol levels in HepaRG cells in a concentration-dependent manner (**Fig. 1A**), consistent with clinical results (6–8). To understand the mechanism by which atRA treatment results in cholesterol accumulation, we analyzed mRNA expression levels of hepatic genes involved in cholesterol homeostasis. The mRNA level of *HMGCR* (the rate-limiting enzyme in cholesterol biosynthesis) was not altered by atRA. On the other hand, the mRNA level of *CYP7A1* (the rate-limiting enzyme in cholesterol elimination) was dramatically decreased upon atRA treatment (Fig. 1B). atRA repressed *CYP7A1* expression at both mRNA and protein levels in a concentration-dependent manner (Fig. 1C). To gauge the importance of CYP7A1 in atRA-induced cholesterol accumulation, we next examined whether increased cholesterol level upon

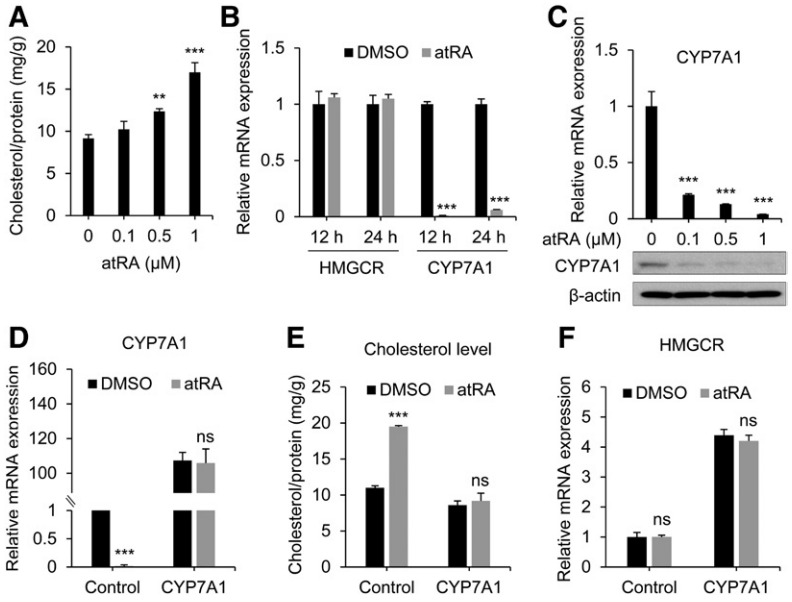

Fig. 1. atRA induces cholesterol accumulation in HepaRG cells. A: HepaRG cells were treated with the indicated concentrations of atRA for 48 h, and total cholesterol levels were measured (n = 3, mean ± SD). ** $P < 0.01$; *** $P < 0.001$ versus control. B: HepaRG cells were treated with atRA (1 μM) for 24 h, and mRNA levels were measured by qPCR (n = 3, mean ± SD). *** $P < 0.001$ versus control. C: HepaRG cells were treated with indicated concentrations of atRA for 48 h, and mRNA (upper) and protein (lower) levels were determined by qPCR and Western blot analysis, respectively. *** $P < 0.001$ versus control. D–F: HepaRG cells were infected with 1×10^7 particles of Flag-CYP7A1 lentivirus (or control) in 6-well plates and treated with atRA (1 μM) or DMSO for 72 h. D, F: mRNA levels were measured by qPCR (n = 3, mean ± SD). *** $P < 0.001$ versus DMSO-treated group. E: Cholesterol levels were measured (n = 3, mean ± SD). *** $P < 0.001$ versus DMSO-treated group. ns, not significant.

atRA treatment can be reversed by *CYP7A1* overexpression. HepaRG cells were infected with lentivirus carrying Flag-CYP7A1, followed by atRA treatment and cholesterol measurement. *CYP7A1* expression was dramatically increased in the infected cells and not affected by atRA treatment (Fig. 1D). Consistently, the basal levels of intracellular cholesterol level were ~20% lower in *CYP7A1*-overexpressing cells and not affected by atRA treatment (Fig. 1E). Of note, *CYP7A1* overexpression led to compensatory increases *in HMGCR* expression (Fig. 1F), potentially accounting for the relatively small decreases in basal cholesterol levels (despite the dramatic increases in CYP7A1 expression in Flag-CYP7A1-infected cells). Similar findings were observed in primary human hepatocytes (supplemental Fig. S1). These results suggest that *CYP7A1* repression contributes to cholesterol accumulation by atRA.

HNF4α is an essential contributor to *CYP7A1* repression by atRA

CYP7A1 mRNA level is known to be regulated at both transcriptional (i.e., promoter activity) and posttranscriptional (i.e., mRNA stability) levels (19). We first examined whether atRA affects mRNA stability of *CYP7A1* by monitoring mRNA decay. The decay rate of *CYP7A1* mRNA was similar between atRA- and DMSO-treated HepaRG cells (supplemental Fig. S2), suggesting that posttranscriptional regulation is not involved in decreased *CYP7A1* mRNA level by atRA.

Multiple response elements for different transcription factors have been reported in the CYP7A1 promoter (**Fig. 2A**) (20). To map the promoter region responsible for CYP7A1 repression by atRA, we generated 5′-deletion constructs of the CYP7A1 promoter and performed promoter reporter assays. To this end, HepG2 cells were used as a transfection host because transfection efficiency in differentiated cells (e.g., primary hepatocytes or HepaRG) is low (42). CYP7A1 repression by atRA disappeared when the −200/−100 region of the CYP7A1 promoter was deleted (Fig. 2B), indicating that the region is critical for atRA response. The region harbors HNF4α/LRH-1 binding sites and putative RARE (DR5) (Fig. 2A). To identify response element(s) responsible for atRA-mediated CYP7A1 repression, we generated mutant constructs for HNF4α/LRH-1 binding sites or DR5 and examined the effect of mutation on atRA response using a promoter reporter assay. Mutation in the LRH-1 binding site or DR5 (M2 and M4, respectively; Fig. 2C) had no effect on atRA response; however, the mutation in the HNF4α binding site (M1 and M3) abrogated atRA-mediated CYP7A1 repression (Fig. 2C). To confirm the role of HNF4α in atRA-mediated CYP7A1 repression, we knocked down HNF4A expression using siRNA and examined its effects on CYP7A1 promoter activity. HNF4α depletion led to decreased CYP7A1 promoter activity at basal levels (Fig. 2D), consistent with the critical role of HNF4α as an activator of the CYP7A1 promoter (13, 23, 24). Of note, it also abrogated atRA-mediated CYP7A1 repression (Fig. 2D). Similar results were obtained upon HNF4A knockdown in human hepatocytes (supplemental Fig. S3). These results indicate that HNF4α (but not LRH-1, RAR, or PXR) is a critical component in the regulatory pathways for atRA-mediated CYP7A1 repression in human liver cells.

To further rule out the involvement of genomic mechanisms in *CYP7A1* repression by atRA, HepaRG cells were treated with RAR and RXR antagonists (ANG193109 and UVI3003, respectively), and *CYP7A1* response to atRA was examined. *CYP26A1* expression was examined as a marker of RAR/RXR action. AGN193109 and UVI3003 decreased atRA-mediated induction of *CYP26A1* (supplemental Fig. S4) as expected; however, these agents had minimal effects on *CYP7A1* repression by atRA, verifying insignificant roles of genomic mechanisms in atRA action on *CYP7A1*.

SHP induction does not play a role in *CYP7A1* repression by atRA

To determine whether *CYP7A1* repression by atRA is mediated via atRA effects on *HNF4A* expression, we measured *HNF4A* mRNA levels following atRA treatment in multiple

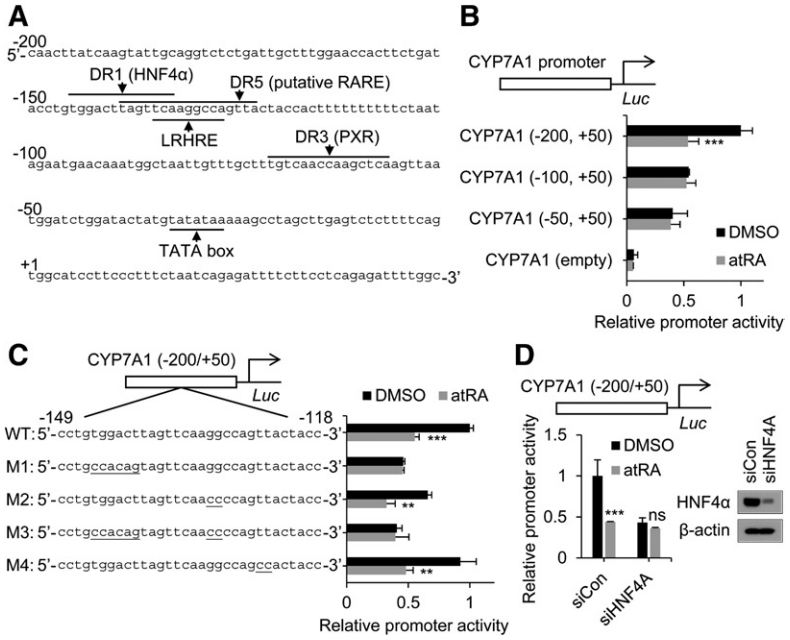

Fig. 2. The HNF4α response element is essential for atRA-mediated *CYP7A1* repression. A: Previously reported hormone response elements in the human *CYP7A1* promoter. B, C: HepG2 cells were transfected with *CYP7A1* promoter luciferase vectors (indicated constructs) and treated with atRA (1 μM), and promoter activity was measured (n = 3, mean ± SD). ** $P < 0.01$; *** $P < 0.001$ versus DMSO-treated group. D: HepG2 cells were transfected with siRNA (20 nM) targeting *HNF4A* (siHNF4A) or control siRNA (siCon). After 24 h, the cells were transfected with *CYP7A1* promoter luciferase vector, followed by treatment with atRA (1 μM) or DMSO for 24 h (n = 3, mean ± SD). Also shown is the Western blot image of the siRNA-transfected cells at 48 h posttransfection. *** $P < 0.001$ versus DMSO-treated group. ns, not significant.

liver cells (i.e., primary human hepatocytes and HepaRG and HepG2 cells). mRNA levels of *CYP26A1*, a marker gene for atRA action, were dramatically increased in all cells (supplementary Fig. S5). Interestingly, whereas *CYP7A1* expression was significantly decreased in all cells, the effects of atRA on *HNF4A* mRNA levels were variable in these cells (i.e., no changes in *HNF4A* expression in HepG2 cells and two of four different batches of human hepatocytes) (supplemental Fig. S5). This result suggests that *CYP7A1* repression by atRA can be mediated by altered HNF4α activity alone (i.e., without changes in its expression).

Transcriptional activity of HNF4α can be modulated by protein interactions with other transcription factors, such as corepressor SHP (29–31). Considering that atRA increases *SHP* expression in the liver (11), we examined whether SHP is required for atRA-mediated *CYP7A1* repression. To this end, we depleted SHP using siRNA transfection in HepaRG cells and human hepatocytes and examined whether it affects *CYP7A1* repression by atRA. atRA increased *SHP* expression in HepaRG cells, in agreement with the previous study (11), and this was abrogated in cells transfected with SHP siRNA (**Fig. 3A–**C). Despite the lack of *SHP* induction, atRA repressed *CYP7A1* expression (Fig. 3D). Similar results were obtained in a batch of human hepatocytes (supplemental Fig. S6). These results suggest that SHP is dispensable for atRA-mediated repression of CYP7A1.

atRA represses *CYP7A1* expression via JNK and ERK activation

atRA activates MAPKs in multiple cell types, including breast cancer cells (33, 43). To determine whether atRA is capable of activating MAPK signaling pathways in liver cells, we treated HepaRG cells and primary human hepatocytes with atRA and detected phosphorylated JNK, ERK, or p38 proteins. Western blot analysis showed that atRA activates all three MAPK pathways in both HepaRG cells and primary human hepatocytes; however, the time profiles of activation for JNK and ERK differed (**Fig. 4A**). For example, ERK activation occurred rapidly (peaking at 15 min) in HepaRG, whereas it gradually increased over time in human hepatocytes (Fig. 4A). To identify MAPK pathways responsible for *CYP7A1* repression by atRA, we treated HepaRG cells with a specific MAPK inhibitor (i.e., SB203580, SP600125, and PD98059 for p38, JNK, and ERK, respectively) and examined atRA-induced *CYP7A1* repression. Treatment with inhibitors completely blocked phosphorylation of respective proteins as expected (supplemental Fig. S7). Although SB203580 had a minimal effect on *CYP7A1* repression by atRA, individual treatment with SP600125 or PD98059 led to partial reversal of atRA action (Fig. 4B). These results indicate the involvement of JNK and ERK pathways in *CYP7A1* regulation by atRA.

AP-1 (c-Jun/c-Fos) binds to the HNF4α binding site to repress *CYP7A1* transcription

AP-1 proteins are downstream targets of JNK and ERK (44). To determine whether AP-1 proteins are involved in *CYP7A1* repression by atRA, we overexpressed AP-1 components (i.e., c-Jun and c-Fos) and examined their effects on *CYP7A1* promoter activity in HepG2 cells. AP-1 overexpression led to a significant decrease in *CYP7A1* promoter activity (**Fig. 5A**, lane 1 vs. 2). atRA treatment in AP-1 overexpressed cells further repressed *CYP7A1* promoter activity (Fig. 5A, lane 2 vs. 4), suggesting enhanced activities of overexpressed AP-1 proteins via phosphorylation.

To explore the possibility that AP-1 proteins directly regulate the *CYP7A1* promoter (not involving HNF4α), promoter reporter assays were performed using luciferase vector harboring the *CYP7A1* promoter with a mutation in its HNF4α response element (M1). The activity of the WT *CYP7A1* promoter was repressed upon overexpression of AP-1 proteins (Fig. 5B, lane 1 vs. 2 or 3 vs. 4); however, it was abrogated when the HNF4α response element in the

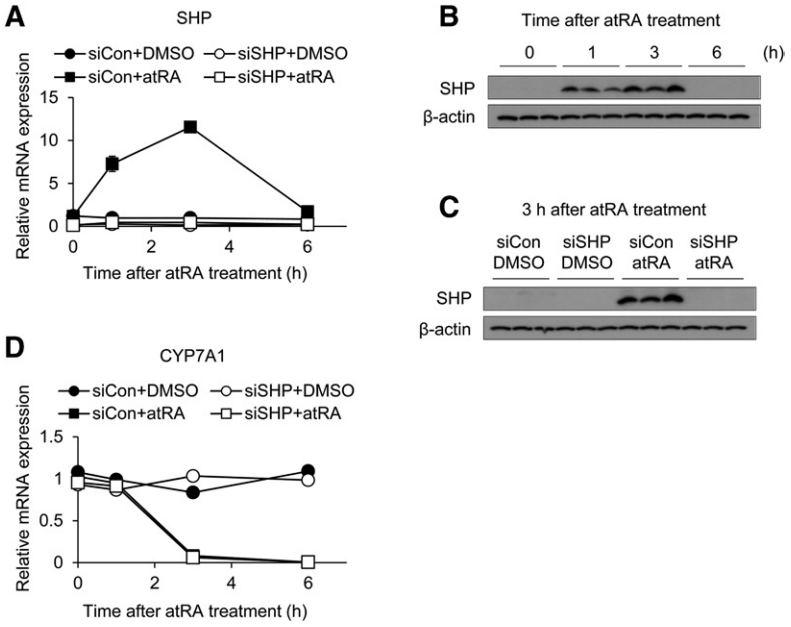

Fig. 3. SHP does not contribute to *CYP7A1* repression by atRA. HepaRG cells were transfected with siRNA (50 nM) targeting *SHP* (siSHP) or control siRNA (siCon), followed by treatment with atRA (1 μM) or DMSO for the time periods indicated. mRNA and protein levels were determined by qPCR (n = 3, mean ± SD) (A, D) and Western blot analysis (B, C), respectively.

CYP7A1 promoter was mutated (Fig. 5B, lane 5 vs. 6 or 7 vs. 8), suggesting that AP-1 action on the *CYP7A1* promoter is HNF4α binding site-dependent. The expression of c-Jun, one of the AP-1 components, was knocked down in HepaRG cells, and *CYP7A1* response to atRA was examined. *CYP7A1* repression by atRA was maintained in c-Jun-knocked-down cells (supplemental Fig. S8), suggesting potential involvement of non-AP-1 mechanisms in atRA action on *CYP7A1* expression.

Next, we examined the recruitment of HNF4α and AP-1 proteins on the *CYP7A1* promoter by DNA pull-down assays. To this end, oligonucleotides harboring HNF4α-binding element of *CYP7A1* (−149/−118) were used. RAR recruitment was examined as a negative control based on the results from promoter reporter assays (Fig. 2C). RAR did not bind to the oligonucleotide, as expected (Fig. 5C). atRA decreased HNF4α and increased c-Jun/c-Fos bound to the DNA. The changes in HNF4α, c-Jun, and c-Fos binding to the oligonucleotide appear partly due to altered expression levels of the proteins in atRA-treated cells. For example, atRA led to a dramatic increase in c-Fos protein levels (Fig. 5C), consistent with a previous report that phosphorylation leads to transactivation of c-Fos promoter and stabilization of c-Fos protein (45–47). The binding of HNF4α, c-Jun, and c-Fos decreased dramatically after the addition of unlabeled WT oligonucleotides, indicating that the proteins bind specifically to the oligonucleotide.

To confirm whether AP-1 recruitment to the DNA is HNF4α-dependent, oligonucleotides harboring mutated HNF4α response element (i.e., m1 and m2) were generated, and DNA pull-down assays were performed. The binding of HNF4α to the m1 and m2 oligonucleotides dramatically decreased, whereas AP-1 proteins did not interact with m1 or m2 oligonucleotides, regardless of atRA treatment (Fig. 5D), suggesting that AP-1 binding to the *CYP7A1* promoter is HNF4α-dependent. ChIP assay in HepaRG cells revealed that the binding of AP-1 to the region of the *CYP7A1* promoter was increased during atRA treatment, whereas the binding of HNF4α was decreased (Fig. 5E), in agreement with the results from the DNA pull-down assay.

Considering that our results indicate the involvement of nongenomic mechanisms in the atRA action, the changes in the recruitments of AP-1 proteins and HNF4α were further examined upon blockade of transcription. HepaRG cells were pretreated with actinomycin D followed by atRA treatment and ChIP assays. Actinomycin D pretreatment

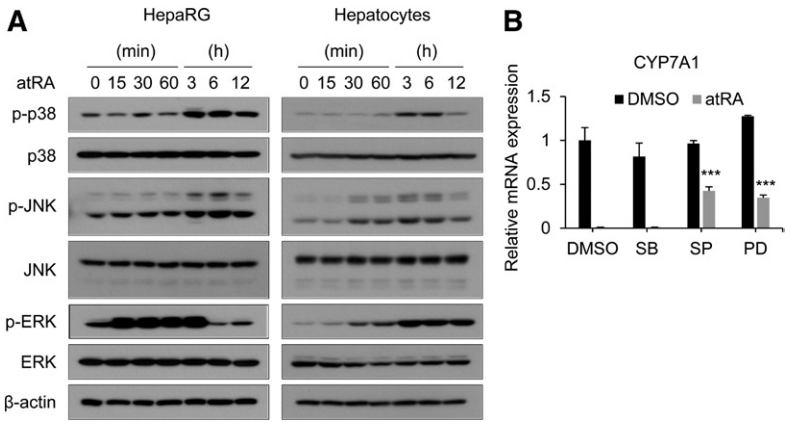

Fig. 4. atRA activates MAPK pathways to regulate *CYP7A1* expression. A: HepaRG cells and human hepatocytes were treated with atRA (1 μM) for the time periods indicated. Protein levels were determined by Western blot analysis. B: mRNA levels of *CYP7A1* in the cells were measured by qPCR. (n = 3, mean ± SD). *** $P < 0.001$ versus DMSO-treated group. PD, PD98059; SB, SB203580; SP, SP600125.

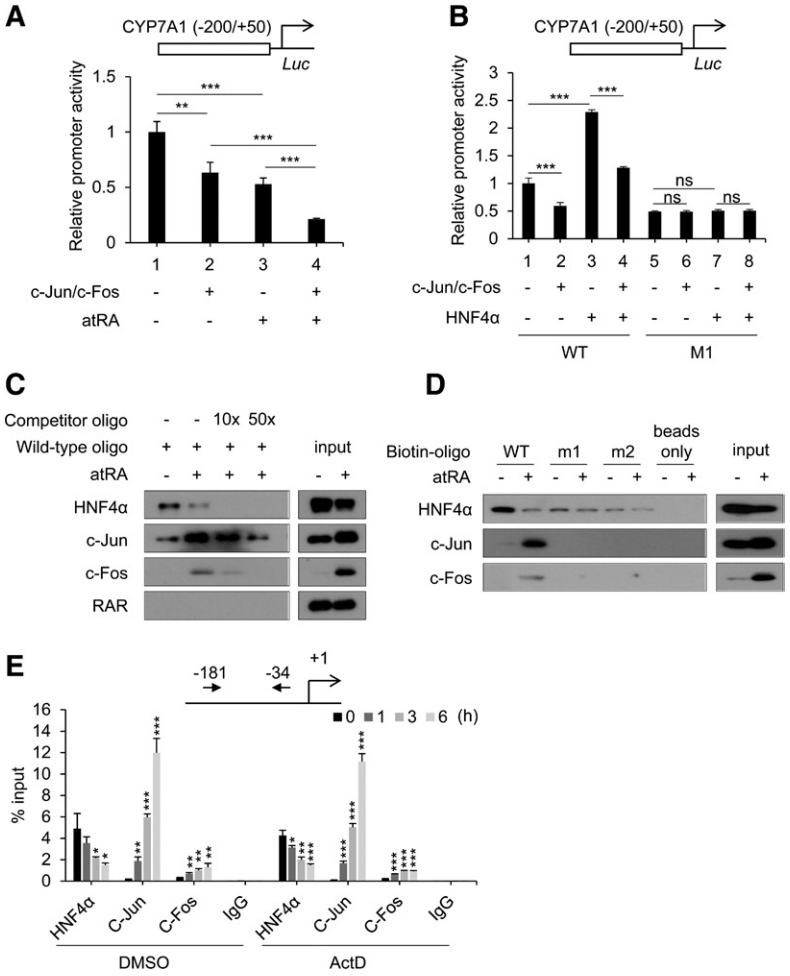

Fig. 5. AP-1 proteins are recruited to the HNF4α-binding site of the *CYP7A1* promoter in a HNF4α-dependent manner. A: HepG2 cells were cotransfected with *CYP7A1* promoter luciferase vector and expression vectors (c-Jun and c-Fos) and treated with atRA (1 μM) for 24 h, and promoter activity was measured (n = 3, mean ± SD). * $P < 0.05$; *** $P < 0.001$. B: HepG2 cells were cotransfected with CYP7A1 promoter luciferase vector and HNF4α expression vectors, and promoter activity was measured (n = 3, mean ± SD). *** $P < 0.001$; ns, not significant. M1, mutated HNF4 binding site. C, D: HepaRG cells were treated with atRA (1 μM) for 3 h. Western blot analysis was performed after DNA pull-down using the biotinylated oligonucleotides indicated and nuclear extracts. E: HepaRG cells were pretreated with Actinomycin D (ActD; 2 μg/ml) for 1 h and treated with atRA (1 μM) for the time periods indicated, and ChIP assay was performed (n = 3, mean ± SD). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control (0 h).

did not affect the temporal changes in AP-1 and HNF4α recruitment to the *CYP7A1* promoter (Fig. 5E). Together, these results indicate that AP-1, upon activation by atRA, decreases HNF4α transactivation of the *CYP7A1* promoter by inhibiting HNF4α recruitment to the promoter.

JNK/ERK signaling pathways play a crucial role in atRA-induced cholesterol accumulation

To further define the roles of JNK and ERK pathways in atRA-induced cholesterol accumulation, we examined the effects of JNK and ERK inhibitors (i.e., SP600125 and PD98059, respectively) on atRA-induced cholesterol accumulation in HepaRG cells. Inhibition of JNK and ERK pathways (**Fig. 6A**) led to significant decreases in atRA-induced *CYP7A1* repression (Fig. 6B) and cholesterol accumulation (Fig. 6C). Although combined treatment with SP600125 and PD98059 failed to completely abrogate atRA-mediated *CYP7A1* repression or cholesterol accumulation, the results suggest that JNK/ERK, AP-1, and HNF4α signaling cascade is a major pathway for atRA repression of *CYP7A1* transcription.

DISCUSSION

Retinoids, including atRA, are commonly used to treat certain cancers or dermatological diseases, but are often accompanied by hypercholesterolemia. The molecular mechanisms underlying atRA-induced hypercholesterolemia remain unclear. In this study, we conducted a thorough investigation of the mechanisms. To this end, liver cells of human origin were used, in consideration of previously reported interspecies differences in the regulation of *CYP7A1* (22, 48). Our results indicate that atRA activates JNK/ERK signaling pathways, and subsequent inhibition of HNF4α transactivation of the *CYP7A1* promoter potentially contributes to hypercholesterolemia by atRA.

The liver is a major organ for the maintenance of cholesterol homeostasis by controlling cholesterol synthesis and elimination. These processes are governed mainly by the actions of two rate-limiting enzymes, HMGCR and CYP7A1. In human hepatocytes and HepaRG cells, atRA had no effects on *HMGCR* expression, but dramatically decreased *CYP7A1* expression at both mRNA and protein levels. Overexpression of *CYP7A1* protected the cells from atRA-induced cholesterol accumulation. These results suggest that hypercholesterolemia by atRA is caused by downregulation of *CYP7A1*, consistent with the idea that CYP7A1 plays a critical role in maintaining cholesterol homeostasis, as previously shown in humans or mice with CYP7A1 deficiency (49, 50).

CYP7A1 expression is regulated at both transcriptional and posttranscriptional levels (51, 52). *CYP7A1* mRNA

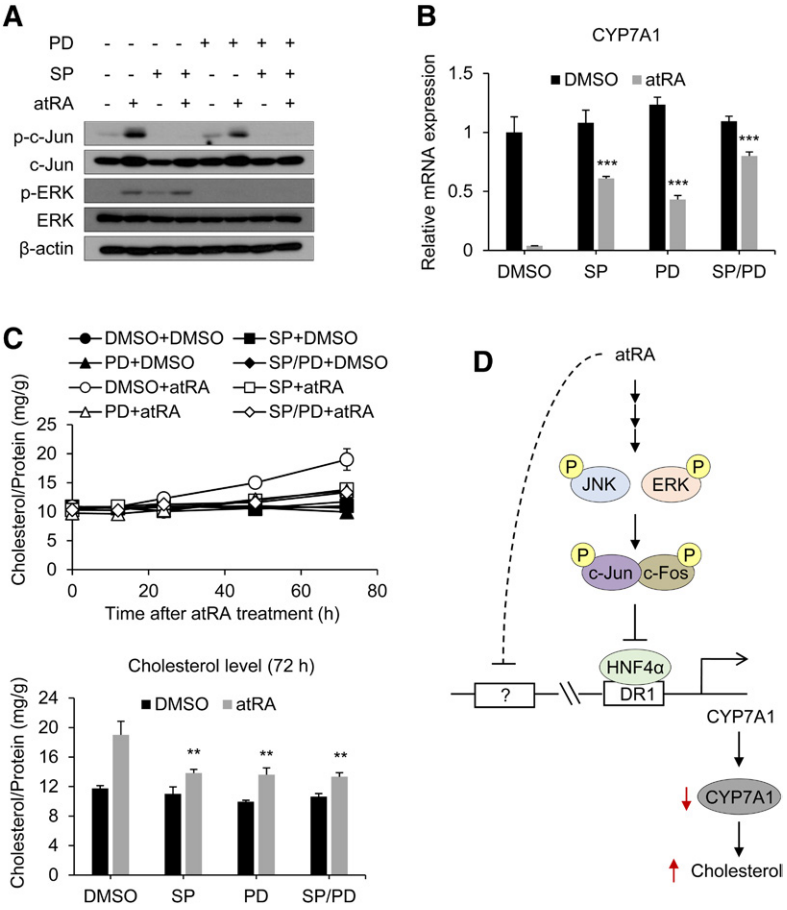

Fig. 6. JNK/ERK inhibition reverses atRA-induced cholesterol accumulation. A, B: HepaRG cells were pretreated with SP600125 (SP; 50 μM) and/or PD98059 (PD; 50 μM) for 1 h and treated with atRA (1 μM) for 3 h. A: Protein levels were determined by Western blot analysis. B: mRNA levels were determined by qPCR (n = 3, mean ± SD). *** $P < 0.001$ versus atRA-treated group. C: HepaRG cells were pretreated with SP600125 (50 μM) and/or PD98059 (50 μM) for 1 h and treated with atRA (1 μM) for the time periods indicated. Total cholesterol levels were measured. Total cholesterol levels at the 72 h time point are presented (n = 3, mean ± SD). ** $P < 0.01$ versus atRA-treated group. D: A model depicting mechanisms for *CYP7A1* repression by atRA is shown.

half-life was not affected by atRA treatment, suggesting that atRA-induced *CYP7A1* repression is likely mediated by its effects on the *CYP7A1* promoter. Indeed, results from promoter reporter assays revealed that atRA decreases *CYP7A1* promoter activity. The human *CYP7A1* promoter harbors at least two AGGTCA-like repeating sequences, providing potential binding sites for nuclear receptors such as HNF4α, LRH-1, RAR, and PXR. Our results from the *CYP7A1* promoter reporter assay using deletion or site-specific mutated constructs indicate that the HNF4α binding site is critical for atRA-mediated *CYP7A1* repression. Moreover, depletion of HNF4α abrogated atRA-mediated *CYP7A1* repression in HepG2 and human hepatocytes, supporting the idea that HNF4α is a critical component in *CYP7A1* regulation by atRA in human liver tissue. These results also suggest that the involvement of other potential transcriptional regulators in *CYP7A1* repression by atRA (e.g., RAR) is minor, if any.

Our results showed minimal effects of atRA on *HNF4A* expression in HepG2 cells and two (out of four) batches of human hepatocytes. Importantly, *CYP7A1* repression by atRA was still observed in these cells, suggesting that atRA action on *CYP7A1* expression occurs mainly through atRA modulating HNF4α activity (rather than *HNF4A* expression). Corepressors, including SHP, are known to interact with HNF4α and alter DNA binding affinity of HNF4α (53–55). atRA was previously shown to induce *SHP* expression in human hepatocytes (11), suggesting the potential roles of SHP in *CYP7A1* repression by atRA. However, *SHP* knockdown (using siRNA) had a minimal effect on atRA-mediated *CYP7A1* repression, indicating minor roles of SHP in atRA-mediated *CYP7A1* repression. This appears in line with previous findings that *SHP* expression was not essential in *CYP7A1* repression in cholestasis (56). Of note, *SHP* is a representative target gene of FXR, a permissive binding partner of RXR. Our findings also suggest that FXR/RXR (in addition to SHP) is a minor player in atRA-induced cholesterol accumulation. This notion is consistent with a previous report where *FXR* knockdown (using siRNA) had no effect on *CYP7A1* repression by atRA in primary human hepatocytes (11).

The rapid changes in HNF4α activity and the lack of actinomycin D effects on the temporal changes in HNF4α recruitment to the *CYP7A1* promoter (Fig. 5E) suggest that the activation is likely mediated by nongenomic actions of atRA. Our results in HepaRG cells indicate important roles of MAPK in altered HNF4α action by atRA. Although atRA activated three MAPK pathways (i.e., ERK, JNK, and p38), the study using pharmacological inhibitors revealed that JNK and ERK signaling pathways (but not p38) are critical in atRA-mediated *CYP7A1* repression. Previous studies have shown that bile acids activate JNK, and its downstream target c-Jun inhibits coactivator binding to HNF4α via direct interaction with HNF4α (28, 57). Our results showed that, similarly to bile acids, atRA activates MAPK pathways. Subsequent activation of AP-1 proteins c-Jun and c-Fos led

to increases in c-Jun and c-Fos binding to the HNF4α response element of *CYP7A1* and decreased *CYP7A1* promoter activity. Together, these results suggest that atRA activation of ERK/JNK and subsequent AP-1 proteins repress HNF4α transactivation of the *CYP7A1* promoter (Fig. 6D).

To evaluate the role of JNK/ERK signaling in atRA-induced cholesterol accumulation, we examined the effects of pharmacological inhibition of JNK/ERK pathways on cholesterol level in atRA-treated HepaRG cells. Individual inhibition of JNK and ERK signaling rescued HepaRG cells from cholesterol accumulation during atRA treatment. Of note, the JNK and ERK inhibitors lacked additive or synergistic effects on cholesterol accumulation, and the combination treatment failed to fully reverse cholesterol accumulation in atRA-treated cells. Additionally, c-Jun knockdown failed to abrogate CYP7A1 repression by atRA, suggesting the presence of additional pathways (not involving MAPK/AP-1) in *CYP7A1* repression by atRA (Fig. 6D). Although our study focused on the promoter region (−200/+50) of *CYP7A1*, other transcription factors, including estrogen-related receptor γ, are known to regulate *CYP7A1* transcription by binding to (∼1.5 kilobase) upstream enhancer region (58). Whether these regulatory factors contribute to atRA-induced cholesterol accumulation in liver cells remains to be investigated.

How atRA triggers the activation of the MAPK pathway remains unclear. Stress signals such as reactive oxygen species (ROS) can activate MAPK signaling pathways, and retinoids were previously shown to cause mitochondrial swelling and decrease mitochondria membrane potential (59), a condition that induces ROS accumulation. Indeed, we found in HepaRG cells that atRA increased ROS level by 2-fold at 1 h after treatment (supplemental Fig. S9). However, inhibition of ROS accumulation by *N*-acetyl-cysteine did not affect atRA-mediated MAPK activation or *CYP7A1* repression (supplemental Fig. S9), suggesting minimal roles of ROS in MAPK activation by atRA.

Conversion to bile acids followed by biliary excretion is a major elimination pathway of cholesterol from the body (60). The dramatic reduction in CYP7A1 expression upon atRA treatment suggests subsequent decreases in biliary elimination of cholesterol as bile acids, and this may trigger compensatory stimulation of other elimination pathways for cholesterol. ABCA1 is a membrane transporter expressed ubiquitously (including liver) and is involved in cholesterol removal from tissues to systemic circulation as HDL (61). ABCG5 and ABCG8, located at the canalicular membranes of hepatocytes, facilitate efflux of cholesterol into bile for excretion into the intestine (60). In HepaRG cells, atRA had minimal effects on ABCA1 expression (supplemental Fig. S10). On the other hand, it decreased ABCG5 and ABCG8 expression by ∼2-fold, potentially due to the repressive effects of atRA on HNF4α action on their promoters (62). Of note, the latter finding suggests the possibility that decreased biliary transport of cholesterol via ABCG5 and ABCG8 may underlie intracellular cholesterol accumulation upon atRA treatment. Interestingly, however, animal studies have shown decreased (rather than increased) hepatic cholesterol levels when Abcg5 and Abcg8 are deleted (60, 63). The roles of ABCG5/8 in atRA effects on hepatic cholesterol accumulation remain to be clarified.

In conclusion, we showed that atRA causes cholesterol accumulation in human liver cells in part by downregulation of *CYP7A1* transcription. This is triggered by activation by ERK/JNK signaling and subsequent downstream effector AP-1 proteins, leading to decreased HNF4α transactivation of the *CYP7A1* promoter. These results provide mechanistic insights into atRA-induced cholesterol disorder.

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