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Identification of 5α,6α-Epoxycholesterol as a Novel Modulator of Liver X Receptor Activity

Thomas J. Berrodin, Qi Shen, Elaine M. Quinet, Matthew R. Yudt, Leonard P. Freedman,[1] and Sunil Nagpal

Women's Health & Musculoskeletal Biology, Wyeth Research, Collegeville, Pennsylvania

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ABSTRACT

The liver X receptors (LXRα and LXRβ) are members of the nuclear receptor superfamily that function as key transcriptional regulators of a number of biological processes, including cholesterol homeostasis, lipid metabolism, and keratinocyte differentiation. Natural ligands that activate LXRs include oxysterol derivatives such as 25-hydroxycholesterol, 27-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 20(*S*)-hydroxycholesterol, and 24(*S*),25-epoxycholesterol. Related oxysterols, such as 5α,6α-epoxycholesterol (5,6-EC) are present in a number of foods and have been shown to induce atherosclerosis in animal models. Intriguingly, these oxysterols have also been detected in atherosclerotic plaques. Using a variety of biochemical and cellular assays, we demonstrate that 5,6-EC is the first dietary modulator and an endogenous LXR ligand with cell and gene context-dependent antagonist, agonist, and inverse agonist activities. In a multiplexed LXR-cofactor peptide interaction assay, 5,6-EC induced the recruitment of a number of cofactor peptides onto both LXRα and LXRβ and showed an EC_{50} of approximately 2 μM in peptide recruitment. Furthermore, 5,6-EC bound to LXRα in a radiolabeled ligand displacement assay (EC_{50} = 76 nM), thus demonstrating it to be one of the most potent natural LXRα ligands known to date. Analysis of endogenous gene expression in various cell-based systems indicated the potential of 5,6-EC to antagonize LXR-mediated gene expression. Furthermore, it also induced the expression of some LXR-responsive genes in keratinocytes. These results clearly demonstrate that 5,6-EC is an LXR modulator that may play a role in the development of lipid disorders, such as atherosclerosis, by antagonizing the agonistic action of endogenous LXR ligands.

Introduction

Liver X receptors (LXRα/NR1H3 and LXRβ/NR1H2) are oxysterol-dependent transcription factors that belong to the steroid/thyroid hormone nuclear receptor superfamily. LXR agonists induce the expression of genes involved in cholesterol efflux and transport and decrease the expression of inflammatory mediators in macrophages and microglia (Chawla et al., 2001; Zelcer and Tontonoz, 2006; Zelcer et al., 2007). Therefore, LXR ligands have the potential to treat atherosclerosis and Alzheimer's disease (Joseph et al., 2002; Tontonoz and Mangelsdorf, 2003; Zelcer et al., 2007). In addition, LXR agonists display potent anti-inflammatory activities, and have shown therapeutic efficacy in murine models of dermatitis and rheumatoid arthritis (Fowler et al., 2003; Chintalacharuvu et al., 2007). Because the cause of photoaging and chronological skin aging involves continuous cutaneous inflammation, LXR has also been identified and validated as a novel target for the prevention and treatment of skin aging (Chang et al., 2008). LXR ligands increase cholesterol efflux by inducing the expression of cholesterol binding (ApoE and ApoD) proteins and cholesterol transporters belonging to the ATP-binding cassette family, namely ABCA1, ABCG1, ABCG5, and ABCG8 (Zelcer and Tontonoz, 2006). In contrast, LXRs mediate their anti-inflammatory effects by antagonizing NF-κB-mediated gene expression (Joseph et al., 2003). The mechanism underlying the anti-NF-κB activity involves the recruitment of sumoylated LXR to the NF-κB motif, which then prevents the degradation of associated nuclear receptor corepressor complexes (Ghisletti et

T.J.B. and Q.S. contributed equally to this work.

[1] Current affiliation: Department of Biochemistry and Molecular Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania.

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ABBREVIATIONS: LXR, liver X receptor; ABCA1, ATP-binding cassette family transporter A1; ACACA, acetyl-coenzyme A carboxylase α; Apo, apolipoprotein; EC, epoxycholesterol; FASN, fatty acid synthase; Ivl, involucrin; KO, knockout; LBD, ligand binding domain; LDLR, low density lipoprotein receptor; MFI, mean fluorescence intensity; NRIP, nuclear receptor interacting protein; OHC, hydroxycholesterol; PLTP, phospholipid transfer protein; SCD, steroyl CoA desaturase; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SRC, steroid receptor coactivator; SREBF, sterol response element binding factor; VDR, vitamin D receptor; wt, wild type; PCR, polymerase chain reaction; FBS, fetal bovine serum; TLDA, TaqMan Low-Density Array; NF-κB, nuclear factor κB; ATCC, American Type Culture Collection; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; GST, glutathione transferase; T0901317, *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide; MTG, monothioglycerol.

al., 2007). A number of natural endogenous oxysterol ligands, namely 24, 25-epoxycholesterol (24,25-EC), 22(*R*)-hydroxycholesterol (22-OHC), 20(*S*)-hydroxycholesterol (20-OHC), 25-hydroxycholesterol (25-OHC), and 27-hydroxycholesterol (27-OHC) that activate LXRs have been identified (Janowski et al., 1996; Lehmann et al., 1997; Fu et al., 2001).

Atherosclerosis is the most common cause of morbidity and mortality in the Western world. Atherosclerotic lesions and early fatty streaks contain cholesterol and its oxygenated derivatives, thus implicating oxysterols in the pathogenesis of atherosclerosis (Staprans et al., 1998, 2000, 2003, 2005, 2006). Oxysterols are also found in foam cells or cholesterol-loaded macrophages that are the drivers of the initial phase of the disease (Hultén et al., 1996). Oxidation of cholesterol by autooxidation, lipid peroxidation, or enzymatic oxidation gives rise to a number of oxysterols. In addition, processing of cholesterol-rich foods, such as meat, egg, and dairy products and dried or stored fish, also produces variable amounts of oxysterols that are consumed by humans. Oxysterols are postulated to be the key proatherogenic components because rabbits fed oxidized cholesterols show more severe focal arterial wall damage and atherosclerotic lesions than those fed nonoxidized cholesterol (Staprans et al., 1998; Brown and Jessup, 1999). Likewise, in a mouse model of atherosclerosis, oxysterols increased the lesion severity in the aorta (Staprans et al., 2000). The pathways and mechanisms by which oxysterols induce the proatherogenic effects are currently not understood. In addition, apart from cardiovascular disease and atherosclerotic lesions, oxysterols have been suspected to be involved in Alzheimer's disease, osteoporosis, and age-related macular degeneration. 5α,6α-Epoxycholesterol (5,6-EC) is among the major oxysterols present in processed foods (Leonarduzzi et al., 2002), which is also detected in human plasma, chylomicrons, and lipoproteins upon feeding oxysterol-rich diets (Gray et al., 1971; Björkhem et al., 1988; Staprans et al., 2005). In addition, serum concentration of 5,6-EC was higher in patients with hypercholesterolemia, and there seems to be a correlation between the serum levels of 5,6-EC and the degree of atherosclerosis (Gray et al., 1971; Björkhem et al., 1988). 5,6-EC is produced by autooxidation of cholesterol and endogenously by enzymatic reaction in the body involving cholesterol-5α,6α-epoxidase (Smith and Johnson, 1989). Here, we demonstrate that 5,6-EC is the first dietary modulator of LXR activity, because it inhibited the agonist-mediated expression of various LXR target genes in multiple cell types and showed agonistic activity on the expression of some of the LXR-responsive genes in keratinocytes. Because LXR agonists have shown efficacy in vivo in models of atherosclerosis, Alzheimer's disease, and inflammation, our results indicate that 5,6-EC may play a role in the development of lipid disorders by antagonizing the agonistic action of the endogenous LXR ligands.

Materials and Methods

Multiplex LXR-Cofactor Peptide Interaction Assay. Each unique fluorescently coded low-capacity avidin modified Luminex bead (RADIX Biosolutions, Georgetown, TX) was incubated overnight with 1 of 39 biotinylated peptides (Anaspec, San Jose, CA) or biotin control at 12.5 μM. The beads were washed twice with multiplex buffer (50 mM Tris, pH 8.0, 50 mM KCl) and incubated with 100 μM D-biotin (Invitrogen, Carlsbad, CA) for 30 min at room temperature to block any remaining unbound avidin sites. After two additional washes, the beads were pooled and resuspended in multiplex buffer to make a 2× concentrated stock containing approximately 40,000 of each unique bead per milliliter. The assay was performed for 2 h at room temperature in 100-μl volume consisting of multiplex buffer, 10 nM GST LXRα ligand binding domain (LBD) protein (amino acids 207–447), or GST LXRβ LBD protein (amino acids 243–461) (Invitrogen), 0.1% bovine serum albumin, 2 mM dithiothreitol, 0.8 μg/ml anti-GST phycoerythrin antibody (Martek, Columbia, MD), and 1× peptide beads along with the test compounds. The plates were read on the Luminex (Austin, TX) instrument using standard settings with a minimum of 50 events quantified for each bead per well. Data were normalized by subtracting the background mean fluorescence intensity, determined with vehicle alone, from the mean fluorescence intensity obtained with each compound treatment.

AlphaScreen Assay. The assays were performed for 2 h at room temperature in 20-μl volume containing 50 mM Tris, pH 8.0, 50 mM KCl, 0.1% bovine serum albumin, 2 mM dithiothreitol, 5 nM GST LXRβ LBD protein (amino acids 207–447), or GST LXRα LBD protein (amino acids 243–461) (Invitrogen), 5 nM biotinylated cofactor peptide (Anaspec), 25 μg/ml anti-GST acceptor, and streptavidin donor AlphaScreen beads (PerkinElmer Life and Analytical Sciences, Waltham, MA). The interaction between the receptor and cofactor peptide in the presence of test compounds was quantified using a PerkinElmer Envision plate reader with the standard AlphaScreen settings. Data were analyzed by a nonlinear regression model using SAS/Excel version 1.03 software (SAS Institute, Cary, NC).

Mammalian Two-Hybrid Assay. The LBDs of LXRα (amino acids 205–448) and LXRβ (amino acids 219–462) were cloned into the GAL4-DBD vector (Clontech, Mountain View, CA). SRC-2 (amino acids 620-1121) was cloned into the pVP16 vector (Clontech). For the GAL4UAS-luciferase reporter, five copies of the 17-base pair GAL4UAS sequence along with the E1b minimal TATA promoter were cloned into the pG5 basic luciferase vector (Promega, Madison, WI). Experiments were performed in COS-7 African green monkey kidney fibroblast-like cells (ATCC, Manassas, VA) grown in phenol red-free Dulbecco's modified minimum essential medium (Invitrogen) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone Laboratories, Logan, UT). The GAL4-LXR LBD fusion plasmid, VP16 SRC-2 fusion plasmid, and the GAL4UAS-luciferase reporter plasmid (50 ng/well each) were transfected for 16 h into COS-7 cells using FuGENE 6 (0.5 μl/well) according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN). Cells were treated with compounds for 20 h, and luciferase activity was measured using Cell Culture Lysis buffer and Luciferase Reagent (Promega, Madison, WI) on a Victor^2 luminometer (PerkinElmer Life and Analytical Sciences). Data were analyzed by the nonlinear regression model using SAS/Excel v1.03 software (SAS Institute).

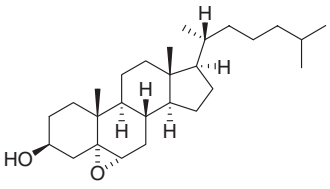
LXR Binding Assay. LXR ligand binding domains with an N-terminal biotinylation tag expressed in *Escherichia coli* were used for the LXR binding assays. For LXRα, amino acid residues 197 to 447 of human LXRα constituted the LBD. For LXRβ, residues 154 to 461 of the human LXRβ constituted the LBD. Cell extracts were made in 50 mM Tris-HCl, pH 7.4 at +4°C, 100 mM KCl, 8.6% glycerol, 0.1 mM PMSF, and 2 mM MTG. Tracer was ^{3}H-labeled *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide (T0901317) at 27 Ci/mmol (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Streptavidin-coated flash plates (SMP 103; PerkinElmer Life and Analytical Sciences) were washed with 100 mM Tris-HCl, pH 7.4 at +4°C, 100 mM KCl, 8.6% glycerol, 0.1 mM PMSF, and 2 mM MTG. Receptor extract was diluted so that 200 μl of diluted extract gave a B_{max} of approximately 4000 cpm. Next, 200 μl/well was added to the plate that was wrapped in aluminum foil and incubated at +4°C over

TABLE 1

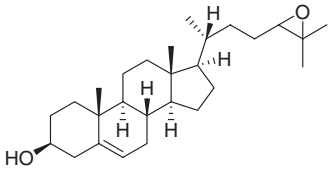
Biotinylated cofactor peptides

Sequence of nuclear receptor cofactor peptides used in the multiplex assay. This table lists the sequences and the National Center for Biotechnology Information reference sequence (RefSeq) accession numbers of the 39 peptides used in the multiplexed cofactor assay.

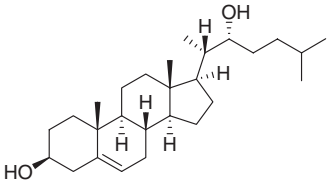
Bead No.	Peptide	RefSeq No.	N-Biotin Peptide Sequences
1	SRC-1 I	NP_003734	KKDSKYSQTSHKLVQLLTTTAEQQR
2	SRC-1 II	NP_003734	HSSLTERHKILHRLLQEGSPSDITT
3	SRC-1 III	NP_003734	KKKESKDHQLLRYLLDKDEKDLRST
4	SRC-1 IV	NP_003734	TQKPTSGPQTPQAQQKSLLQQLLTE
5	SRC-2 I	NP_006531	RLHDSKGQTKLLQLLTTKSDQMEPS
6	SRC-2 II	NP_006531	GTSLKEKHKILHRLLQDSSSPVDLA
7	SRC-2 III	NP_006531	VSPKKKENALLRYLLDKDDTKDIGL
8	SRC-3 I	NP_858045	GPLESKGHKKLLQLLTCSSDDRGHS
9	SRC-3 II	NP_858045	GSLLQEKHRILHKLLQNGNSPAEVA
10	SRC-3 III	NP_858045	SPKKKENNALLRYLLDRDDPSDALS
11	DRIP205 I	NP_004765	KKDFSKVSQNPILTSLLQITGNGG
12	DRIP205 II	NP_004765	MAGNTKNHPMLMNLLKDNPAQDFST
13	PGC-1a	NP_037393	PPQEAEEPSLLKKLLLAPANTQLSY
14	PGC-1b I	NP_573570	KKPAPEVDELSLLQKLLLATSYPT
15	PGC-1b II	NP_573570	KKSKASWAEFSILRELLAQDVLCDVSK
16	CBP I	NP_004371	VPDAASKHKQLSELLRGGSGSSINP
17	CBP/p300 II	NP_004371	PEKRKLIQQQLVLLLHAHKCQRREQ
18	CBP III	NP_004371	QPPRSISPSALQDLLRTLKSPSSPQ
19	p300 I	NP_001420	VQDAASKHKQLSELLRSGSSPNLNM
20	p300 II	NP_001420	LKPGTVSQQALQNLLRTLRSPSSPL
21	PCAF	NP_003875	DADTKQVYFYLFKLLRKSILQRGKP
22	NCOR1 I	NP_006302	KKGKTTITAANFIDVIITRQIASDK
23	NCOR1 II	NP_006302	KKTHRLITLADHICQIITQDFARN
24	NCOR1 III	NP_006302	KKSFADPASNLGLEDIIRKALMGS
25	SMRT I	NP_006303	HQRVVTLAQHISEVITQDYTRHHPQ
26	SMRT II	NP_006303	VQEHASTNMGLEAIIRKALMGKYDQ
27	NRIP1 I	NP_003480	VHQDSIVLTYLEGLLMHQAAGGSGTKK
28	NRIP1 II	NP_003480	VPKGKQDSTLLASLLQSFSSRLQTV
29	NRIP1 III	NP_003480	LRCYGVASSHLKTLLKKSKVKDQKP
30	NRIP1 IV	NP_003480	SPKPSVACSQLALLLSSEAHLQQYSKK
31	NRIP1 V	NP_003480	NIKQAANNSLLLHLLKSQTIPKPMN
32	NRIP1 VI	NP_003480	SKLNSHQKVTLLQLLLGHKNEENVE
33	NRIP1 VII	NP_003480	IENLLERRTVLQLLLGNPNKGKSEK
34	NRIP1 VIII	NP_003480	QDFSFSKNGLLSRLLRQNQDSYLAD
35	NRIP1 IX	NP_003480	WARESKSFNVLKQLLLSENCVRDLS
36	ARA70 I	NP_005428	EETLQQQAQQLYSLLGQFNCLTHQLKK
37	ARA70 II	NP_005428	ENGSRETSEKFKLLFQSYNVNDWLV
38	MNAR I	NP_055204	PATMELAVAVLRDLLRYAAQLPALFKK
39	MNAR II	NP_055204	ALFRDISMNHLPGLLTSLLGLRPEC
40	Biotin	Control	



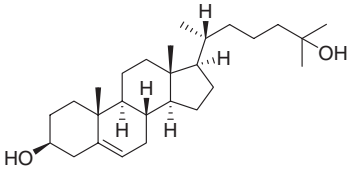
5,6-EC



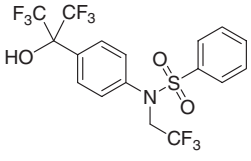
24,25-EC



22-OHC



25-OHC



T0901317

Fig. 1. Chemical structures of 5,6-EC, 24,25-EC, 22-OHC, 25-OHC, and T0901317.

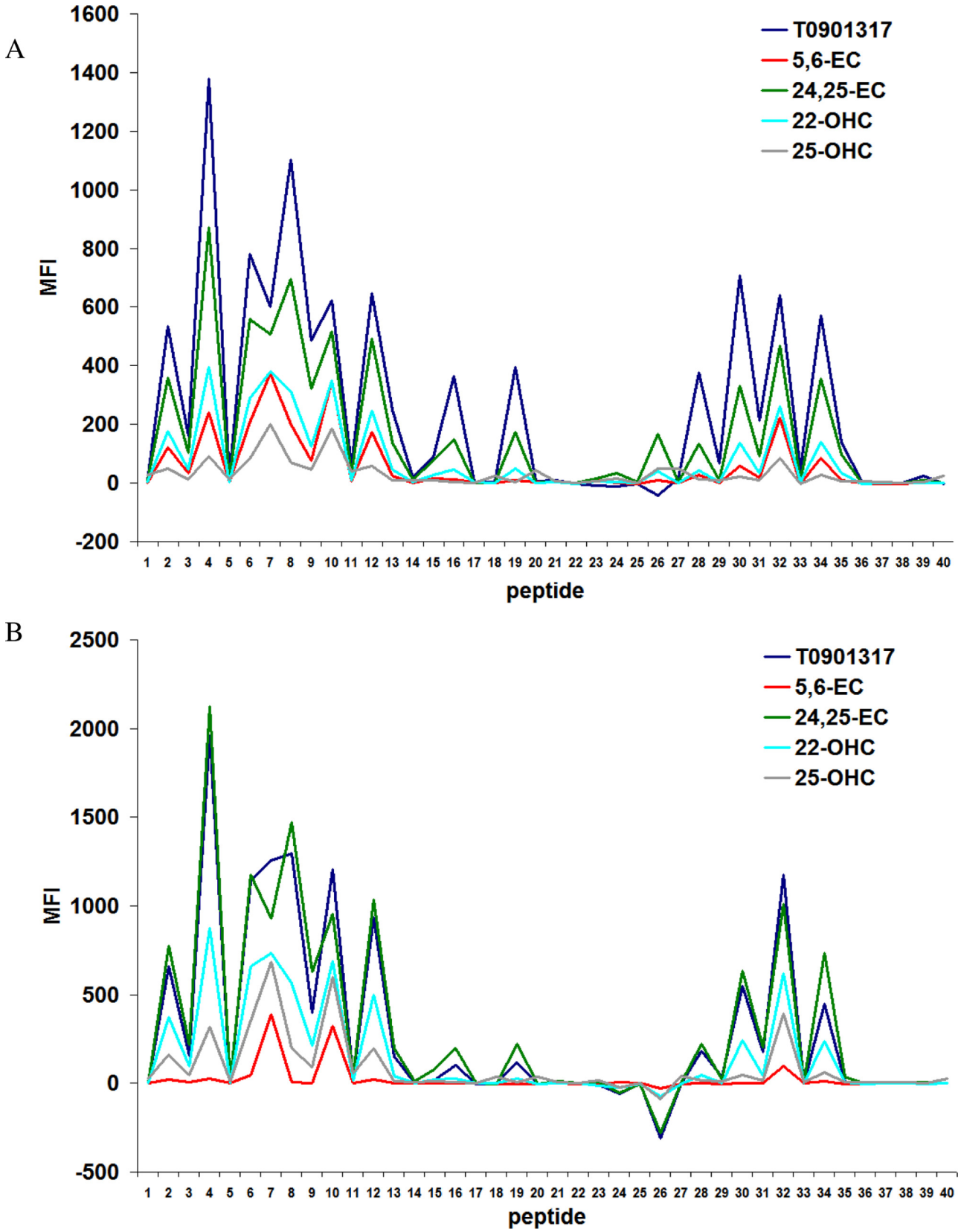


Fig. 2. Characterization of 5α,6α-EC and known LXR ligands in the multiplexed cofactor interaction assay. The interactions of purified 10 nM GST-tagged LXRα (A) or LXRβ (B) LBDs with 39 cofactor peptides were analyzed in a multiplexed assay using Luminex beads. The LXR LBDs were incubated with the cofactor peptide beads, as listed in Table 1, along with 10 μM ligand for 2 h at room temperature. The data are represented as the MFI in the presence of each ligand, subtracting the background mean fluorescence intensity produced in the presence of dimethyl sulfoxide vehicle alone.

night. A dilution series of reference or test compound in 5 nM tracer solution was made in Costar C3365 polypropylene microtiter plates (Corning Life Sciences, Acton, MA). Assay buffer was 100 mM Tris-HCl, pH 7.4 at +4°C, 100 mM KCl, 8.6% glycerol, 0.1 mM PMSF, 2 mM MTG, and 0.2% CHAPS. LXR-coated flash plates were washed three times with assay buffer without CHAPS, and residual buffer was aspirated thoroughly. Test compound (200 μl) in 5 nM tracer was added to each well. Final concentrations of test compound ranged from 100 μM to 50 pM, and each concentration of test compound was analyzed in duplicate. Plates were wrapped in aluminum foil and incubated overnight at +4°C. Test compound/tracer mix was aspirated, and plates were washed once with assay buffer without CHAPS. Finally, residual liquid was aspirated, and plates were sealed according to instructions from the manufacturer. Radioactivity was measured in a Wallac Microbeta normalized for flash plates (PerkinElmer Life and Analytical Sciences). Data were analyzed by the nonlinear regression model using XLfit software (ID Business Solutions, Guildford, Surrey, UK).

Cell Culture. Human hepatoma cell line Huh-7 and human lung adenocarcinoma epithelial cell line A549 were purchased from ATCC. Huh-7 cells were maintained in minimal essential medium/F-12 and Dulbecco's modified Eagle's medium (3:1) with 10% FBS, and A549 cells were cultured in low-glucose Dulbecco's modified Eagle's medium with 10% FBS. In general, cells were seeded on day 0, treatment as designed was done on day 1, and cells were harvested on day 2 for RNA isolation using RNeasy column (QIAGEN, Hilden, Germany). Gene expression profiles were analyzed using TaqMan Low-Density Array (TLDA) and individual TaqMan gene assays (Applied Biosystems, Foster City, CA).

THP-1 cells (ATCC) were maintained in RPMI 1640 medium (Invitrogen) containing 10% FBS, 2 mM L-glutamine, and 55 μM β-mercaptoethanol. Confluent THP-1 cells were treated with 50 to 100 ng/ml phorbol 12,13-dibutyrate (Sigma-Aldrich, St. Louis, MO) suspended in ethanol for 3 days to induce differentiation into adherent macrophages. LXR ligands dissolved in ethanol (EtOH) were added to differentiated cells for 18 h, then total cellular RNA was isolated using a PrepStation 6100 (Applied Biosystems) according to the manufacturer's recommendations. RNA concentrations measured with RiboGreen assay (Invitrogen), and hABCA1 mRNA were quantified by real-time PCR analysis as described previously (Quinet et al., 2004). For antagonism studies, the T0901317 agonist was dosed at its EC_{50} concentration.

LXR-β Wild-Type and KO Skin Cell Preparations. LXR-β KO mice were obtained from Deltagen (San Carlos, CA) in the 129 strain

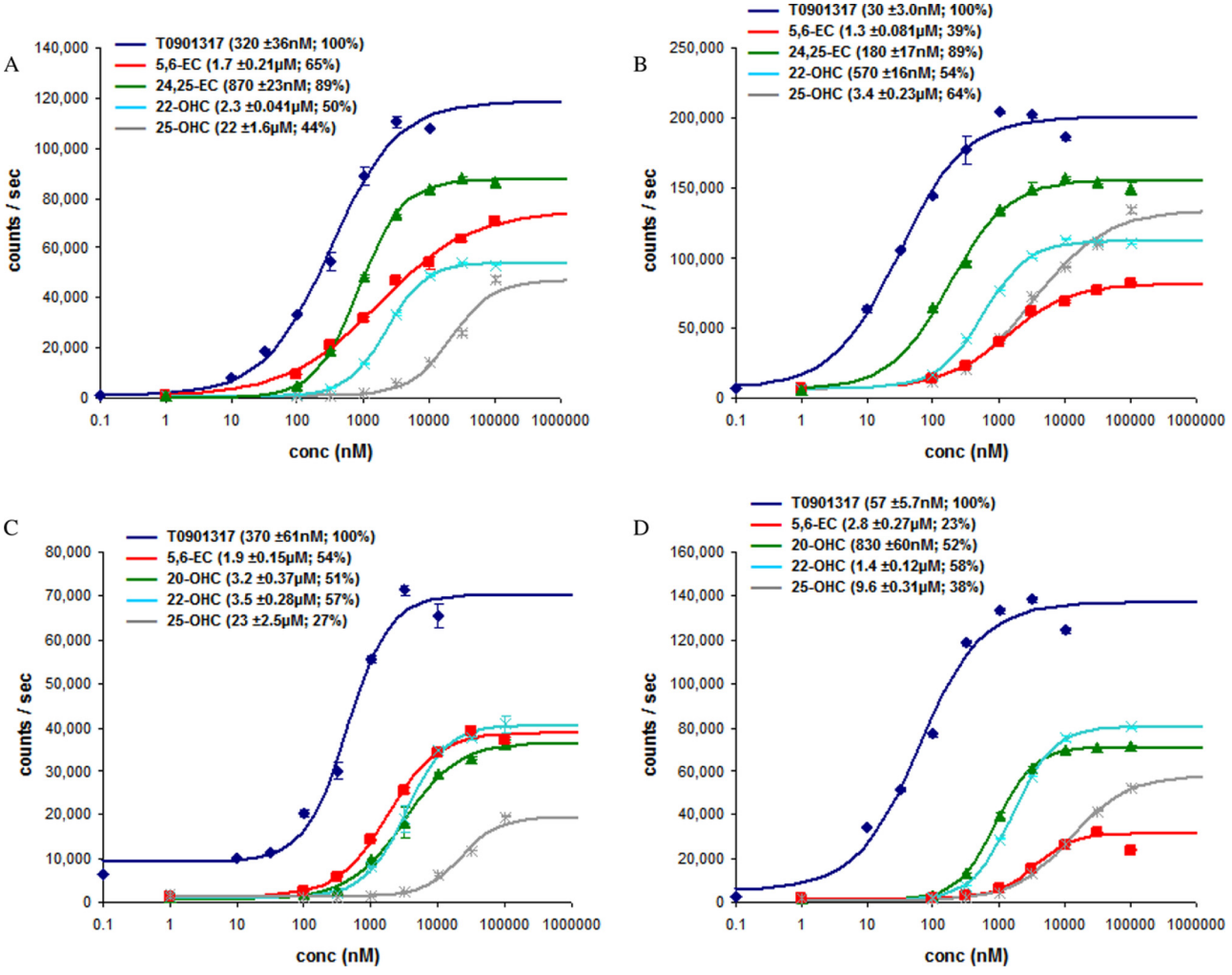


Fig. 3. Dose-response analysis of LXR ligands using pairwise LXR/cofactor interaction assays. The interaction of purified 5 nM GST-tagged LXRα (A and C) or LXRβ (B and D) LBDs with 5 nM concentration of the coactivator peptides SRC-2 III (A and B) or SRC-3 III (C and D) were measured using AlphaScreen beads. The LXR LBDs were incubated with cofactor peptides, AlphaScreen beads, and serial dilutions of ligands for 2 h. The data were fitted to a four-parameter nonlinear logistic model to determine the EC_{50} values for each compound. The efficacy was calculated as a percentage of the maximal activity induced by T0901317. Dose-response curves for T0901317 (diamonds with dark blue lines), 5,6-EC (squares with red lines), 24,25-EC (triangles with green lines), 22-OHC (crosses with light blue lines), and 25-OHC (asterisks with gray lines) are shown. The results for each compound are reported in parentheses: (EC_{50} ± S.E.; efficacy).

and backcrossed for seven generations into black C57BL/6J mice. LXR-β KO was accomplished using LXR-β gene sequence deletion from base 226 to 395 by using a homologous recombination vector (Deltagen). Skins from newborn mice (2–3 days old) were isolated and floated on 2.5 mg/ml dispase overnight at 4°C and separated into epidermal and dermal layers using small forceps. The epidermal and dermal layers were minced and subjected to several differential centrifugations, fractionations and filtrations as described previously (Zheng et al., 2005). These cells were then cultured in Eagle's minimal essential medium containing fetal bovine serum (8%) in 24-well culture plates (day 0). Cells were treated as described on day 2, followed by isolation and purification of RNA on day 3 using RNeasy column.

Custom-Designed TLDAs and Quantitative Reverse-Transcriptase Polymerase Chain Reaction. The RNA obtained from the compound treated cells were used in custom-designed TLDAs or individual TaqMan assays (Applied Biosystems) according to the manufacturer's protocols using ABI 7900HT real-time PCR machine. The level of expression was calculated based on the PCR cycle number (Ct), and the relative gene expression level was determined using $\Delta\Delta$Ct method as described elsewhere. One TLDA was designed with oligonucleotide probes and primer pairs for involucrin (Ivl) (Mm00515219_s1), Abca1 (Mm00442646_m1), ApoE (Mm00437573_m1), Scd1 (Mm00772290_m1), Cyp27a1(Mm00470430_m1), ABCA1 (Hs00194045_m1), ACACA (Hs00167385_m1), FASN (Hs00188012_m1), LDLR (Hs00181192_m1), PLTP (Hs00272126_m1), SLC2A4 (Hs00168966_m1), SREBF1 (Hs00231674_m1), ABCG1 (Hs00245154_m1), and SCD1 (Hs00748952_s1).

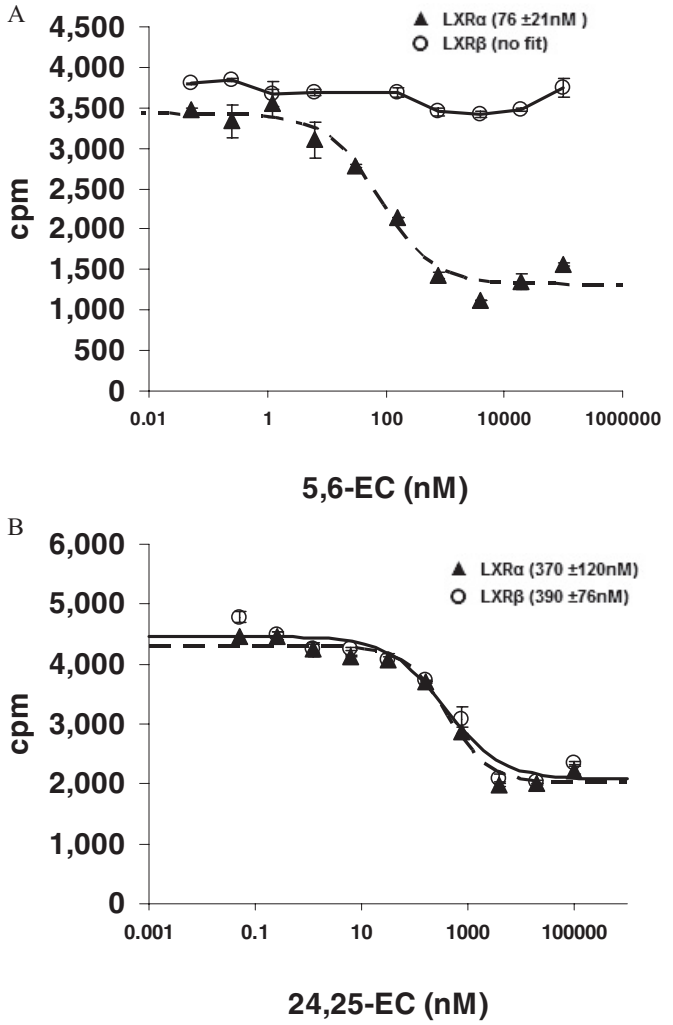


Fig. 4. Comparison of 5,6-EC and 24,25-EC in a competition LXR binding assay. Competition by 5,6-EC (A) and 24,25-EC (B) for 5 nM [^{3}H]T0901317 binding to biotinylated LXRα and LXRβ LBD proteins was measured. IC_{50} values were calculated using a four-parameter nonlinear logistic model. The results are reported in parentheses as IC_{50} ± S.E.

Results

5,6-EC Recruits Cofactor Peptides to LXR LBD Proteins. Nuclear receptors, including LXRs, activate gene expression by ligand-dependent interaction with coactivators that act by modifying chromatin structure and mediating interaction with components of the basal transcription machinery. It has been proposed that NR modulators function in a tissue-selective manner by inducing unique receptor conformations that lead to differential recruitment of coactivators. These coactivators are specifically recruited to nuclear receptors via their LXXLL modules, and small peptides containing LXXLL motifs are sufficient to bind to the receptor in

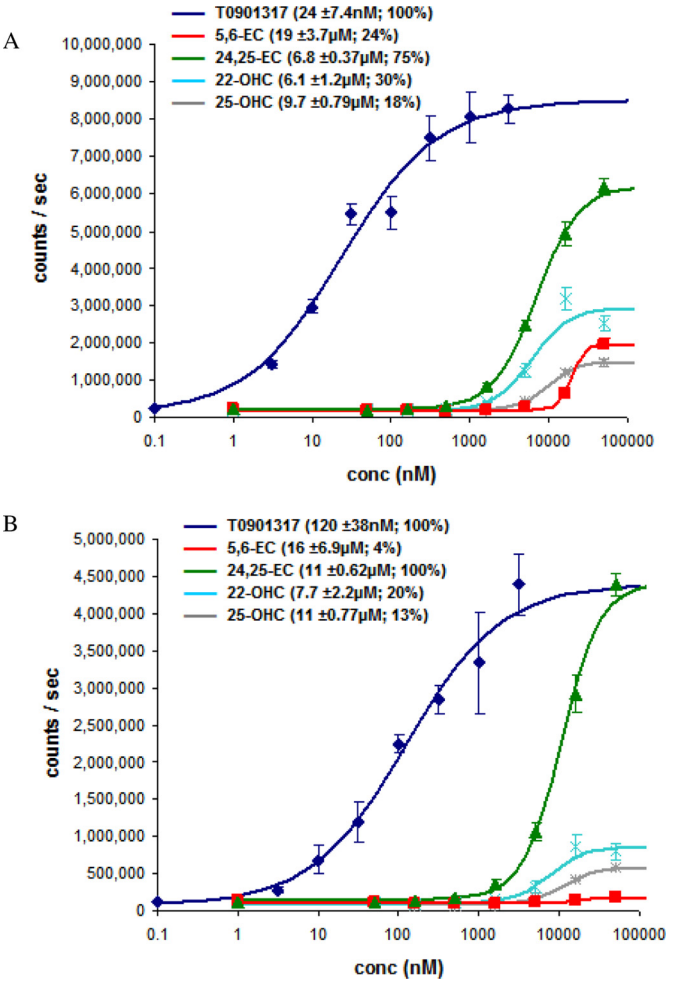


Fig. 5. The activity of LXR ligands in a cell-based mammalian two-hybrid assay. To assess the activity of LXR ligands in cells, COS-7 cells were cotransfected with GAL4-LXR LBD, VP16-SRC-2, and a 5×-GALUAS luciferase reporter. The dose-response curves for the ligands with LXRα (A) and LXRβ (B) are shown. The data were fitted to a four-parameter nonlinear logistic model to determine the EC_{50} values for each compound. The efficacy was calculated as a percentage of the maximal activity induced by T0901317. Dose-response curves for T0901317 (diamonds with dark blue lines), 5,6-EC (squares with red lines), 24,25-EC (triangles with green lines), 22-OHC (crosses with light blue lines), and 25-OHC (asterisks with gray lines) are shown. The results for each compound are reported in parentheses as (EC_{50} ± S.E.; efficacy).

a ligand-dependent manner (Heery et al., 1997; Savkur et al., 2004). Because various oxysterols have been identified as LXR ligands, we decided to examine whether 5,6-EC is also an LXR ligand by analyzing the recruitment of 39 cofactor peptides (Berrodin et al., 2009) to LXRα and β LBDs in a biochemical microsphere-based multiplex NR-cofactor interaction assay (Iannone et al., 2004; Berrodin et al., 2009). The sequences of cofactor peptides used herein are shown in Table 1. The structure of 5,6-EC and the structures of known natural and synthetic LXR ligands is shown (Fig. 1). A saturating concentration (10 μM) of 5,6-EC was used to compare its cofactor recruitment profile with that of other known synthetic and natural oxysterol LXR ligands. The ligand-dependent recruitment of cofactor peptides to the LXRα or β LBDs is expressed as mean fluorescence intensity (MFI) units, which is directly proportional to the affinity of the peptides to the receptor LBD protein. As shown in Fig. 1A, the synthetic LXR ligand T0901317 (Schultz et al., 2000) showed robust interaction with SRC-1II, SRC-1IV, SRC-2II, SRC2-III, SRC-3, vitamin D receptor interacting protein 205 II, PGC-1α, cAMP response element-binding protein binding protein I, p300 I, and various NRIP1 peptides. However, a number of cofactor peptides did not show any binding to LXRα-LBD, and the most potent natural endogenous ligand, 24,25-EC, was less efficacious than T0901317 in recruiting most of the peptides to the receptor LBD (Fig. 2A). It is noteworthy that T0901317 and 24,25-EC induced distinct receptor conformations because the former mediated the dissociation of LXRα-LBD-SMRT II peptide interaction, whereas the later induced the recruitment of the corepressor peptide to the LXRα LBD protein (Fig. 2A). 22-OHC was approximately 50% less efficacious than 24,25-EC but still showed interaction with a similar set of peptides. It is noteworthy that 5,6-EC was less efficacious than T0901317 and 24,25-EC but exhibited efficacy similar to that of 22-OHC on SRC-2 III, SRC-3 III, vitamin D receptor interacting protein 205 II, and NRIP1 VI peptides (Fig. 2A). However, unlike the other natural and synthetic LXR ligands (except for 25-OHC), 5,6-EC did not mediate LXRα-LBD interaction with cAMP response element-binding protein binding protein I, p300 I, SMRT II, and NRIP1 II peptides. The interaction profile revealed that 5,6-EC engendered a conformational change in the LXRα-LBD, which was different from that mediated by the T0901317 and other oxysterol natural ligands. 25-OHC was the least efficacious of all in this assay (Fig. 2A).

In interaction assay with LXRβ-LBD, T0901317 and 24,25-OHC showed similar profiles and were equally efficacious in mediating interaction with similar set of peptides (Fig. 2B). 22-OHC and 25-OHC showed similar cofactor peptide interaction patterns (Fig. 2B). It is noteworthy that 5,6-EC exhibited distinct cofactor peptide interaction profile and mediated the recruitment of only three cofactor peptides, namely SRC-2 III, SRC-3 III, and NRIP1 VI (Fig. 2B). In addition, 5,6-EC was the least efficacious ligand on LXRβ-LBD (Fig. 2B). Furthermore, all of the other ligands except for 5,6-EC showed the dissociation of SMRT II corepressor peptide from

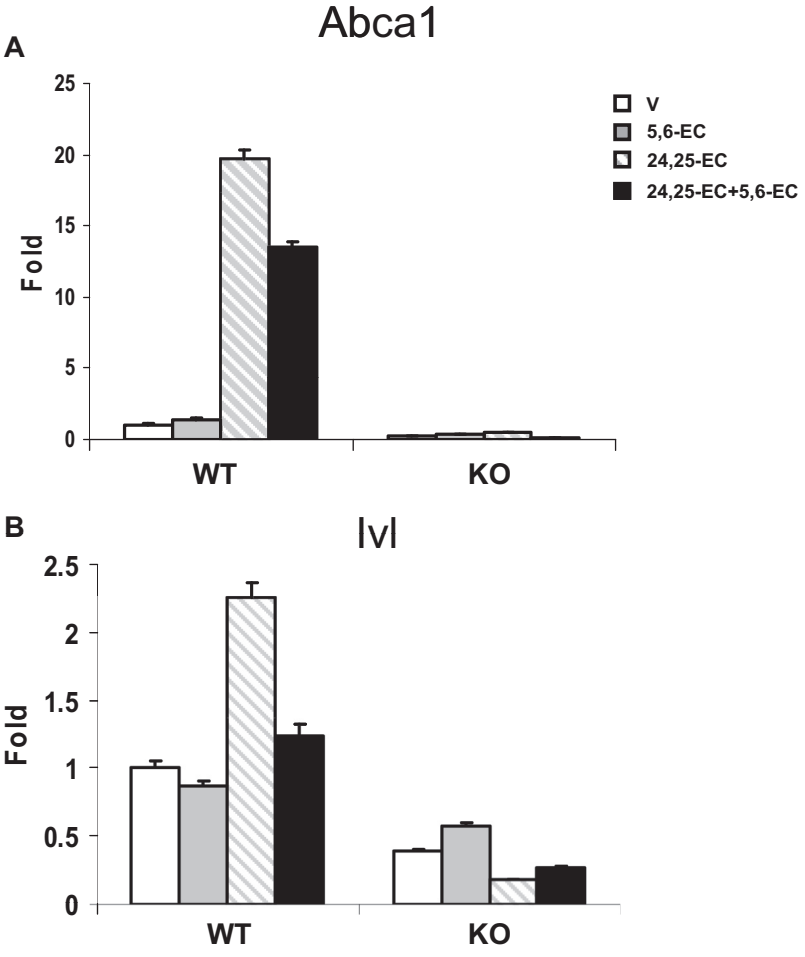


Fig. 6. 5,6-EC antagonizes 24,25-EC-induced Abca1 and Ivl expression in wild-type primary murine keratinocytes. Primary keratinocytes were isolated from wild-type or LXRβ KO newborn mice skin. Cells were treated for 24 h with vehicle (□), 5,6-EC (10 μM; ▨), 24,25-EC (10 μM; ▨) or cotreated with 5,6-EC and 24,25-EC (10 μM each; ■). The relative expression level of Abca1 (A) and Ivl (B) was measured by real-time PCR.

the LXRβ-LBD protein. These results indicate that 5,6-EC induces LXRβ-LBD conformation, which is different from that engendered by the other ligands.

In a biochemical Alphascreen-based dose-dependent LXRα-SRC-2 III recruitment assay, 5,6-EC was nearly as efficacious as 24,25-EC and showed EC_{50} value of 1.7 μM. T0901317, 24,25-EC, 22-OHC, and 25-OHC showed EC_{50} values of 0.32, 0.87, 2.3, and 22 μM, respectively (Fig. 3A). In LXRβ-SRC-2 III interaction assay, T0901317, 5,6-EC, 24,25-EC, 22-OHC, and 25-OHC showed EC_{50} values of 0.03, 1.3, 0.18, 0.57, and 3.4 μM, respectively (Fig. 3B). Likewise, in LXR-SRC-3 III cofactor recruitment assays, 5,6-EC showed EC_{50} values of 1.9 and 2.8 μM for LXRα and LXRβ, respectively (Fig. 3, C and D). In LXRα-SRC-3 III recruitment assay, 5,6-EC was 50% less efficacious than T0901317, whereas it showed efficacy and potency equivalent to that of 20-OHC and 22R-OHC (Fig. 3C). These results clearly demonstrate that 5,6-EC shows single-digit μM EC_{50} values in cofactor-dependent LXRα and β ligand-sensing assays, and it exhibits greater efficacy with LXRα than LXRβ LBD compared with T0901317.

5,6-EC Binds to LXRα and Mediates Cofactor Recruitment in Cells. A ligand competition assay was used to assess the direct binding of 5,6-EC using recombinant human LXRα and β LBD proteins and ^{3}H-labeled T0901317 as a tracer (Hu et al., 2006). Sigmoidal competition curve was obtained for the binding of 5,6-EC to LXRα LBD, but no competition of ^{3}H-labeled T0901317 was observed in LXRβ LBD-based binding assays. 5,6-EC showed IC_{50} value of 76 nM for LXRα in the ligand competition assay (Fig. 4A). However, it failed to significantly displace the radiolabeled synthetic LXR agonist in LXRβ binding assay (Fig. 4A). In contrast, 24,25-EC showed IC_{50} values of 370 and 390 nM, respectively, for LXRα and LXRβ LBDs (Fig. 4B). These results indicate that 5,6-EC binds directly to the LXRα-LBD and exhibits 5-fold more potency than 24,25-EC. In addition, 5,6-EC is unable to displace the radiolabeled T0901317 from the LXRβ-LBD protein.

To determine whether the binding of 5,6-EC to LXR recruits coactivator SRC-2 in a cell-based system, a mammalian two-hybrid system with Gal4-LXRα or β and VP16-SRC-2 expression vectors was used. 5,6-EC showed a dose-dependent recruitment of VP16-SRC-2 to LXRα but not LXRβ-LBD (Fig. 5). In LXRα-SRC-2 mammalian two-hybrid assay, 5,6-EC showed partial agonism (compared with T0901317 and 24,25-EC) and was also the least potent ligand among all of the LXR agonists tested. Moreover, 5,6-EC showed 75 and 67%, respectively, less efficacy than T09013167 and 24,25-EC. In contrast, 5,6-EC failed to mediate LXRβ-LBD-SRC2 interaction, whereas all of the other ligands tested were active. T0901317 and 24,25-EC were equipotent in mediating LXRβ-SRC-2 interaction (Fig. 5). These results indicate that 5,6-EC may function as an antagonist and inhibit agonist-mediated expression of the LXR-responsive genes.

5,6-EC Is a Modulator of LXR-Mediated Gene Expression in Primary Murine Keratinocytes. Multiplex LXR cofactor interaction, AlphaScreen and binding assays indicated that 5,6-EC could bind to LXRs. However, in the absence of VP16-SRC-2, it did not induce Gal4-LXRα/β-mediated activation of a Gal4-responsive reporter (data not shown). Therefore, we next examined whether 5,6-EC could act as an antagonist and analyzed its effect on the expression of known LXR-responsive genes in primary mouse keratinocytes. In these cells, 5,6-EC did not induce the expression of Abca1 or Ivl but effectively antagonized the 24,25-EC-mediated expression of these genes. The 24,25-EC agonism and 5,6-EC antagonism effects were not observed in LXRβ KO keratinocytes (Fig. 6, A and B). We also analyzed the effect of 5,6-EC on ApoE, Scd1 and Cyp27a1, which are also known LXR-responsive genes. 5,6-EC inhibited the basal level expression, whereas T0901317 and 24,25-EC induced the expression of ApoE. 5,6-EC completely antagonized the T0901317- and 24,25-EC-dependent expression in wild-type (wt) keratinocytes. The agonist effects of T0901317 and 24,25-EC and the antagonist effects of 5,6-EC were not observed in LXRβ KO keratinocytes, thus demonstrating the specificity of the LXR agonist and that of 5,6-EC action (Fig. 7A). In contrast to its effect on Abca1, Ivl, and ApoE, 5,6-EC augmented the expression of Scd1 in wt but not in LXRβ KO keratinocytes (Fig. 7B). Scd1 expression was also induced by T0901317 but not by 24,25-EC in keratinocytes. Note that 5,6-EC was a partial agonist on keratinocyte Scd1 expression compared with

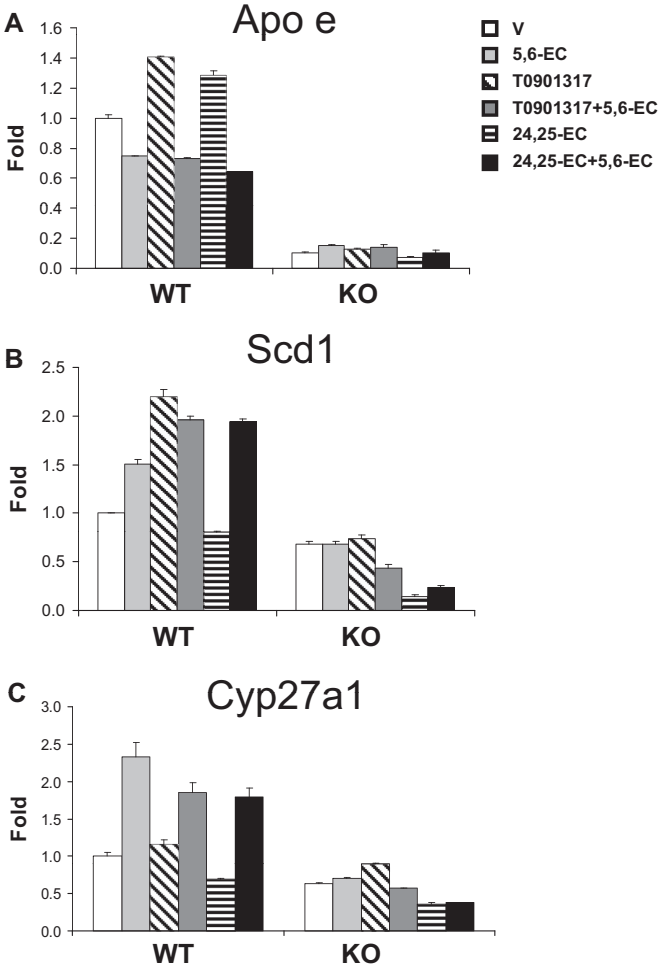


Fig. 7. Effects of 5,6-EC and other ligands on the expression of LXR-responsive genes in primary murine keratinocytes. Primary keratinocytes were isolated from wt or LXRβ KO newborn mice skin. Cells were treated for 24 h with vehicle (open bars), 5,6-EC (10 μM; light gray bars), T0901317 (1 μM; hatched bars), 5,6-EC + T0901317 (5,6-EC 10 μM and T0901317 1 μM; dark gray bars), 24,25-EC (10 μM; striped bars), or 5,6-EC + 24,25-EC (10 μM each; black bars). The relative expression level of ApoE (A), Scd1 (B), and Cyp27a1 (C) was measured by real-time PCR.

T0901317 (Fig. 7B). In contrast to other responsive genes, we show that Cyp27a1 expression was induced by 5,6-EC and not by other classic LXR ligands in wild-type cells but not in LXRβ KO keratinocytes (Fig. 7C). These results indicate that in keratinocytes, 5,6-EC is a gene-selective modulator of LXR action because it could act as an inverse agonist, partial agonist, full agonist, or antagonist of LXR-mediated gene expression.

5,6-EC Is an Antagonist of LXR Agonist-Mediated Gene Expression in Huh-7 and A549 Cells. We also examined the effect of 5,6-EC on the expression of classic LXR-responsive genes in Huh-7 (human hepatocarcinoma) and A549 (human lung carcinoma) cells in agonist and antagonist modes in the presence of the synthetic LXR agonist T0901317. 5,6-EC decreased the basal level expression of SLC2A4 and FASN, and antagonized the T0901317-mediated expression of these genes in Huh-7 cells (Fig. 8, A and C). On the other hand, 5,6-EC did not significantly affect the basal level expression of SREBF1, PLTP, ACACA, and LDLR (Fig. 8, B and D–F). However, it significantly antagonized the T0901317-mediated expression of these genes (Fig. 8, A–F). Likewise, 5,6-EC was an antagonist in A549 cells, because it inhibited the T0901317-induced expression of ABCA1, ABCG1, ACACA, FASN, SCD1, and LDLR (Fig. 9, A–F). Note that 5,6-EC induced the expression of SCD1 in keratinocytes (Fig. 7B) but not in A549 (Fig. 9E), thus indicating that 5,6-EC is a cell context-dependent modulator of LXR-mediated gene expression.

5,6-EC Is a Partial Agonist in Differentiated THP-1 Macrophages. In differentiated THP-1 macrophages, T0901317 and 24,25-EC induced the expression of ABCA1 in a dose-dependent manner with EC_{50} values of 0.024 and 7.7 μM, respectively (Fig. 10A). In contrast, 5,6-EC was a partial agonist on ABCA1 gene expression in THP-1 cells, and it showed only 23% efficacy (relative to T0901317) when tested at the maximum concentration of 30 μM (Fig. 10A). It is noteworthy that unlike in keratinocytes (Fig. 6A), 5,6-EC did not significantly affect T0901317-induced expression of ABCA1 gene in THP-1 macrophages, thus highlighting the cell context-dependent antagonism activity of 5,6-EC (Fig. 10B). Accordingly, 5,6-EC would not be expected to inhibit T0901317-mediated cholesterol efflux in macrophages. Note that 5,6-EC induced ABCA1 expression only in THP-1 macrophages (Fig. 10A) but not in keratinocytes, Huh-7, or A549 cells, further indicating the cell context-dependent activity of 5,6-EC.

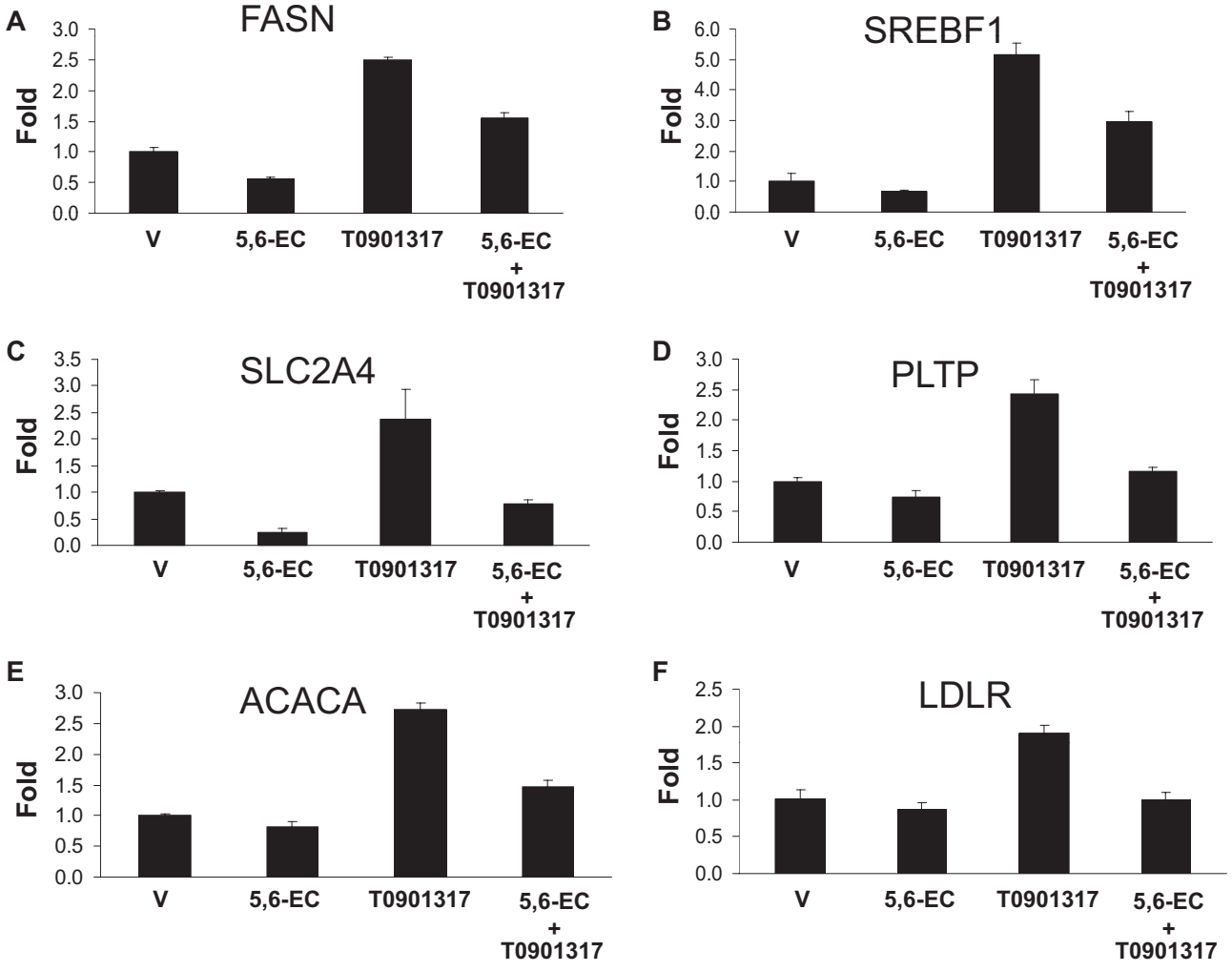


Fig. 8. Antagonism of LXR-mediated gene expression by 5,6-EC in Huh-7 cells. Huh-7 cells were treated with LXR ligands (5,6-EC at 10 μM and T0901317 at 1 μM) for 24 h as indicated in the figure. The relative expression level of FASN (A), SREBF1 (B), SLC2A4 (C), PLTP (D), ACACA (E), and LDLR (F) was measured by real-time PCR.

Discussion

This article describes for the first time that 5,6-EC is a dietary and endogenous modulator of LXR activity. Oxidized cholesterol has been implicated in the pathophysiology of atherosclerosis (Staprans et al., 1998, 2000, 2003, 2005; Vine et al., 1998; Leonarduzzi et al., 2002; Hu et al., 2006). However, the mechanism by which oxidized cholesterol species might provoke oxidative insult is not understood. Cholesterol undergoes rapid autooxidation, peroxidation, and photooxidation, giving rise to various oxidized derivatives, namely 5,6-EC, 7-ketocholesterol, and 25-OHC, which have been identified in human plasma (Gray et al., 1971; Brown and Jessup, 1999). Various oxidized cholesterol derivatives have been detected in lipids extracted from atheromatous aortic lesions (Brown and Jessup, 1999), and oxidation products of cholesterol are known to increase the severity of fatty streak lesions in rabbit and mouse models of atherosclerosis (Staprans et al., 1998, 2000). Animal food products (milk, eggs, meat, and their products) are the major source of cholesterol in our diet. Processing of foods and oils involving high temperature and prolonged storage has been reported to induce the oxidation of cholesterol. Other chemical species such as hydroxyradicals, ozone, oxygen cations, singlet oxygen, superoxide, and peroxides could also aid in the autooxidation of cholesterol (Kumar and Singhal, 1991). Therefore, 5,6-EC and other oxygenated cholesterol derivatives could be generated in the food products before consumption and could also result from the oxidation of endogenous cholesterol. In addition, cholesterol loading of human monocyte-derived macrophages has shown the production of 5,6-EC in quantities comparable with that of 27-OHC (Fu et al., 2001). Although there is no direct proof linking oxidized cholesterol to atherosclerosis in humans, a plethora of in vitro and in vivo studies have highlighted the atherogenic potential of diets rich in oxidized cholesterol (Gray et al., 1971; Kumar and Singhal, 1991; Staprans et al., 1998, 2000). In fact, in humans, dietary 5,6-EC after a single meal is absorbed and incorporated into the pool of oxidized lipids in chylomicrons and endogenous (very low-density lipoprotein, low-density lipoprotein, and high-density lipoprotein) lipoproteins. Furthermore, after a single dose, 5,6-EC remained in circulation with lipoproteins for 72 h, and an increase in oxidized cholesterol content in low density lipoprotein is associated with increased susceptibility of these particles to undergo further oxidation, thus resulting in a larger pool of oxidized cholesterol derivatives (Staprans et al., 2003). These observations indicate that oxidized cholesterol derivatives, including 5,6-EC, may contribute to the pathogenesis of atherosclerosis.

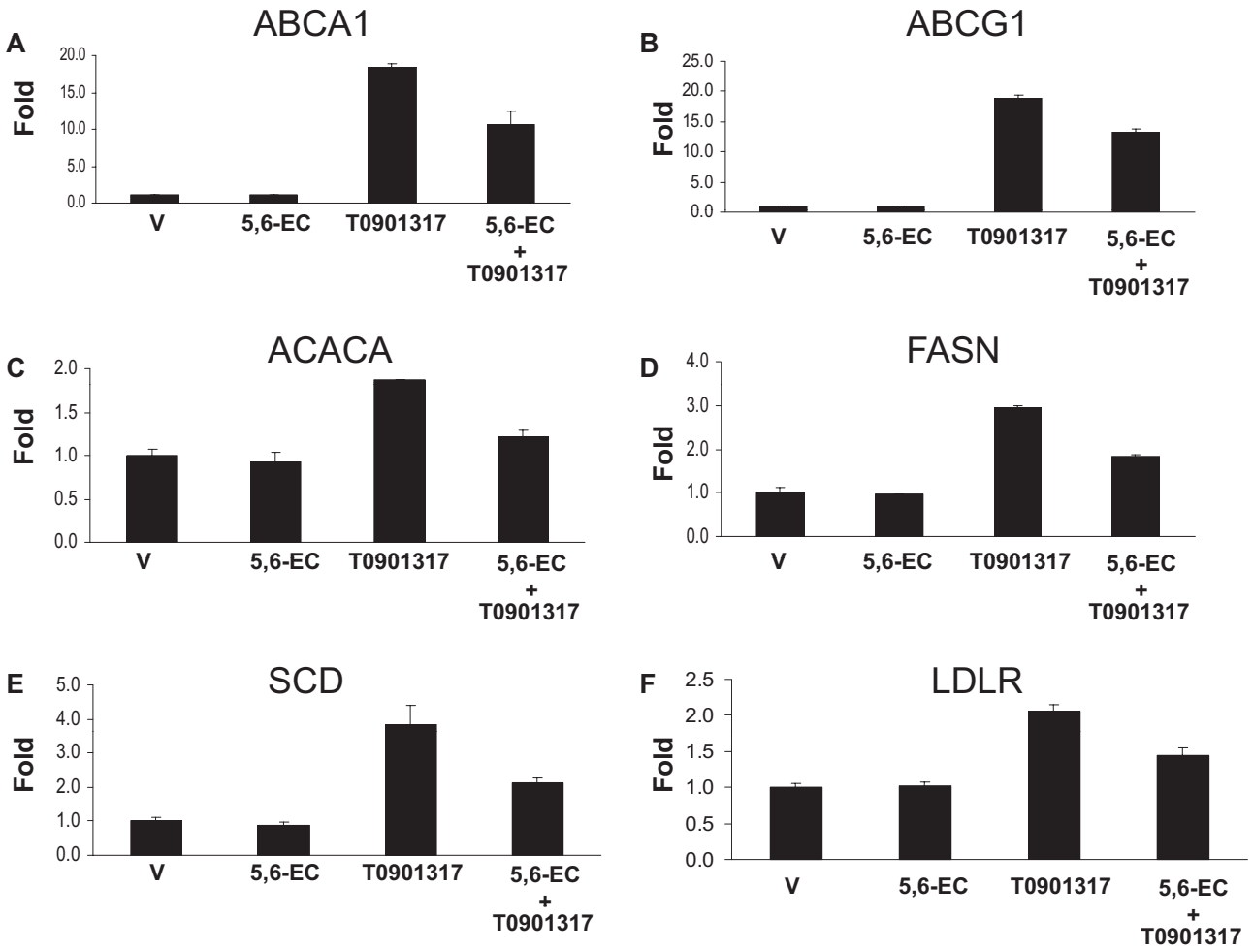


Fig. 9. Antagonism of LXR-mediated gene expression by 5,6-EC in A549 cells. A549 cells were treated with LXR ligands (5,6-EC at 10 μM and T0901317 at 1 μM) for 24 h as indicated. The relative expression level of ABCA1 (A), ABCG1 (B), ACACA (C), FSAN (D), SCD1 (E), and LDLR (F) was measured by real-time PCR.

Here, we show that 5,6-EC (Fig. 1) is an LXR modulator because it mediated the interaction of various coactivator peptides to LXRα and β LBD proteins (Fig. 2), showed dose-dependent interaction of coactivator peptides with the LXR in biochemical assays (Fig. 3), exhibited binding to LXRα-LBD in a ligand competition assay (Fig. 4), and modulated the expression of various LXR-responsive genes in keratinocyte, liver, and lung cells (Figs. 6–9). 5,6-EC inhibited the binding of ^{3}H-labeled T0901317 to LXRα with an EC_{50} value of 76 nM, thus indicating it to be a potent LXRα ligand (Fig. 4). However, it failed to show the displacement of the radiolabeled ligand in the presence of LXRβ LBD in the ligand competition assay (Fig. 4). The interaction of cofactor peptides to LXRα and β LBD proteins (Figs. 2 and 3) and the LXRβ-dependent modulation of gene expression in murine keratinocytes (Fig. 6) suggest 5,6-EC to be a bona fide ligand of LXRα and β isoforms. The differential action of 5,6-EC in displacing [^{3}H]T0901317 from the LXR LBDs could result from high K_{on} and low K_{off} rates of the radiolabeled synthetic ligand on the LXRβ subtype. This phenomenon has been observed in the case of vitamin D receptor (VDR) ligands, where potent VDR agonists did not displace radiolabeled 1,25-dihydroxyvitamin D3 because of its high K_{on} and low K_{off} for the receptor (Boehm et al., 1999; Ma et al., 2006). Our biochemical AlphaScreen-based assays using purified LXRα and β LBD proteins and synthetic cofactor LXXLL motif peptide (SRC-2 NR box III) clearly demonstrate that 5,6-EC is bona fide ligand of both LXRα and β subtypes, with at least a single-digit micromolar potency (Fig. 3). 5,6-EC showed comparable efficacy and potency to oxysterol natural known LXR ligands in LXRα-SRC-2 and SRC-3 peptide interaction assays, and it also mediated LXRβ-LBD interaction with SRC-2/SRC-3, albeit with potency lower than that of 24,25-EC, 22(R)-OHC and 20(S)-OHC (Fig. 3). However, it showed significantly better potency than 25-OHC in mediating the interaction of LXXLL peptides onto LXRα and β LBDs (Fig. 3). Taken together, these results clearly indicate that 5,6-EC is a novel ligand for both LXR subtypes.

Herein, our results demonstrate that 5,6-EC is a novel LXR modulator with cell and gene context-dependent activities (Figs. 5–10). It has been postulated that the tissue- and gene-selective activities of nuclear receptor modulators are due to their abilities to induce distinct conformational changes in the LBD of the receptor, which may result in differential recruitment of cofactors required for the regulation of target gene expression. In the multiplex cofactor peptide interaction assays, 5,6-EC showed distinct profiles of LXRα and LXRβ LBD-peptide interactions that were different from that obtained with T0901317 and known oxysterol LXR ligands (Fig. 1). These results clearly indicate that 5,6-EC induces LXR-LBD conformations that are different from that engendered by T0901317, 22-OHC, 25-OHC and

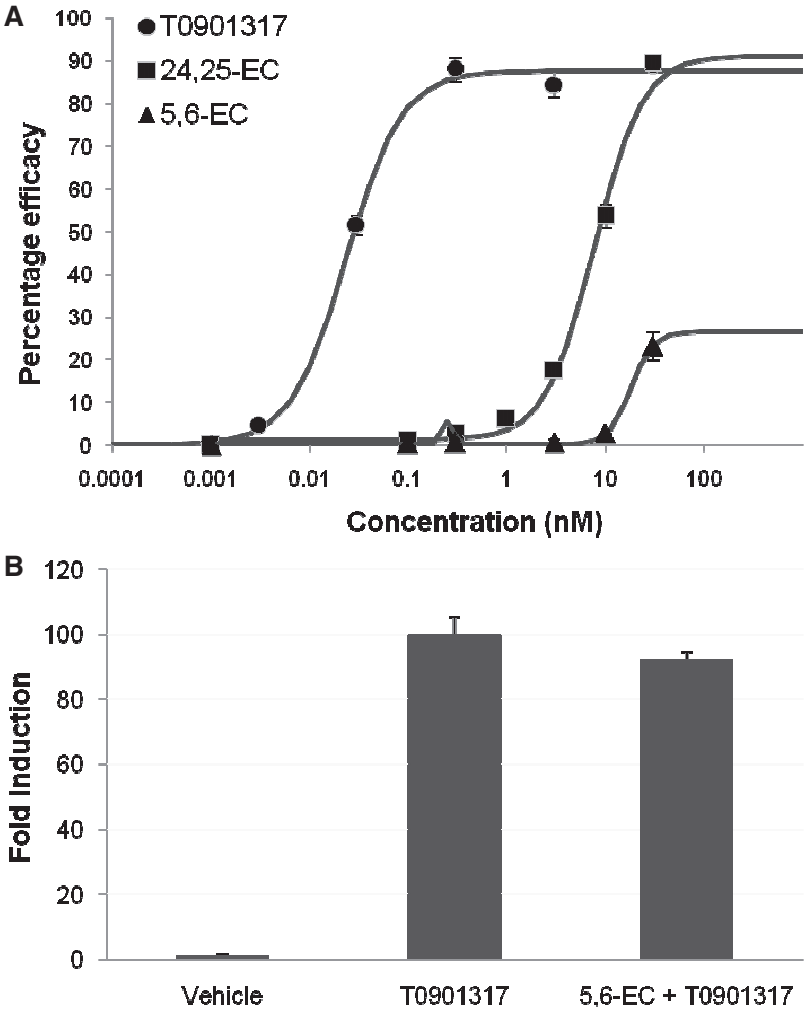


Fig. 10. 5,6-EC is a partial agonist of ABCA1 expression in THP-1 macrophages. Differentiated THP-1 macrophages were treated with varying concentrations of T0901317 (●), 24,25-EC (■) or 5,6-EC (▲) for 18 h. Percentage expression of ABCA1 obtained by the three LXR ligands relative to that obtained by T0901317 (10 μM) is shown in A. Differentiated THP-1 macrophages were treated with vehicle (V), T0901317 (45 nM), or T0901317 (45 nM) + 5,6-EC (30 μM). The expression of ABCA1 is presented as the fold change (relative to vehicle treatment) in B. ABCA1 expression was measured by real-time PCR.

24,25-OHC. The ligand-mediated differential conformations of the receptor may result in differential cofactor recruitment, leading to cell context-dependent activity as observed with noncalcemic vitamin D receptor modulators (Ma et al., 2006). Furthermore, enhanceosome level interaction between LXR with other interacting transcription factors in addition to differential cofactor recruitment may also explain the cell/gene context-dependent activation of gene expression. Such an interaction and its effect on lineage-specific transcription have been described for ERα with FOXA1.

LXR is a key regulator of cholesterol homeostasis. Agonist ligands of LXR induce the expression of genes involved in cholesterol efflux and transport (ABCA1, ABCG1, ABCG5, ABCG8, ApoE, and ApoD) and also decrease the expression of key mediators of inflammation (Chawla et al., 2001; Zelcer and Tontonoz, 2006). These activities are the basis of the efficacious effects of LXR ligands in murine model of atherosclerosis and Alzheimer's disease (Joseph et al., 2002; Tontonoz and Mangelsdorf, 2003; Zelcer et al., 2007). LXR ligands also display potent anti-inflammatory activities in keratinocyte, fibroblast, and immune cell systems, and as a result show efficacy in murine models of atopic and contact dermatitis, rheumatoid arthritis, and photoaging (Fowler et al., 2003; Chintalacharuvu et al., 2007; Chang et al., 2008). In addition, LXR ligands are potent keratinocyte differentiation agents and are also involved in the maintenance of epidermal barrier function by regulating lipid production in keratinocytes (Kömüves et al., 2002; Man et al., 2006; Chang et al., 2008). Therefore, LXR as a target has wide-ranging therapeutic potential in atherosclerosis, arthritis, dermatitis, psoriasis, and skin aging. The discovery reported herein of a natural dietary and endogenous LXR modulator that antagonizes the action of key genes involved in cholesterol transport, lipogenesis, and keratinocyte differentiation is significant. Our results provide a mechanism by which oxidized cholesterol derivatives may induce the formation and progression of atherosclerotic lesions. To the best of our knowledge, 5,6-EC is the first food processing-related modulator of any nuclear receptor. Our results also may explain the underlying reason for the high incidence of atherosclerosis and cardiovascular events observed in Indian/Asian immigrant population in Western countries, which could not be explained by major predisposing factors of hypertension, smoking, intake of red meat, serum cholesterol, and alcohol consumption. One of the risk factors unique to this population may be the ingestion of exceptionally high quantities of oxidized cholesterol (Jacobson, 1987). For example, it has been reported that clarified butter or ghee used as cooking oil medium may be a causative factor for the high incidence of cardiovascular diseases in this population, because it contains a number of cholesterol oxidation products, including 5,6-EC (Jacobson, 1987). Therefore, the identification of 5,6-EC as an antagonist of LXR-mediated gene expression provides a molecular level explanation of the above-mentioned epidemiological observations. Although 5,6-EC did not antagonize T0901317 action on ABCA1 expression in THP-1 macrophages (Fig. 10B), we can not rule out the possibility that it may antagonize the action of endogenous weaker LXR oxysterol ligands in vivo.

In summary, we have identified 5,6-EC as the first novel dietary and endogenous modulator of LXR activity. 5,6-EC is also the most potent natural ligand known for LXRα subtype as assessed by ligand competition binding assay. It mainly functions as an antagonist on a number of classic LXR-responsive genes but, in addition, shows agonist and inverse agonist activities in a cell and gene context-dependent manner. It will be an important tool in further elucidating the physiological role of LXRs and oxysterols in cholesterol/lipid homeostasis, atherosclerosis, inflammation, keratinocyte differentiation, and epidermal barrier formation.

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Address correspondence to: Dr. Sunil Nagpal, Merck Research Laboratories, 770 Sumneytown Pike, West Point, PA 19846-0004. E-mail: sunil.nagpal@merck.com