Bioorganic & Medicinal Chemistry Letters 23 (2013) 579–583

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Induction of ABCA1 and ABCG1 expression by the liver X receptor modulator cineole in macrophages

Hee-jin Jun †, Minh-Hien Hoang †, Siok-Koon Yeo, Yaoyao Jia, Sung-Joon Lee *

Department of Biotechnology, Graduate School of Life Sciences and Biotechnology, Korea University, Seoul 136-713, South Korea

ARTICLE INFO

Article history:
Received 18 July 2012
Revised 16 October 2012
Accepted 6 November 2012
Available online 15 November 2012

Keywords:
Cineole
Liver X receptor
Cholesterol
Macrophage
Hepatocytes

ABSTRACT

We investigated the effect of cineole on the expression of genes related to reverse cholesterol transport and hepatic fatty acid metabolism. Cineole, a small aroma compound in teas and herbs, significantly stimulated the transactivation of liver X receptor modulator (LXR)-α and LXR-β. The mRNA and protein expression of LXRs and their target genes, including ABCA1 and ABCG1, was significantly increased in macrophages stimulated with cineole. This led to the subsequent removal of cholesterol from the cells. Interestingly, cineole showed tissue-selective LXR induction: hepatocytes stimulated with cineole showed significantly reduced expression of LXR-α and LXR-α-responsive genes, including FAS and SCD-1 (P <0.05). Accordingly, hepatocytes treated with cineole displayed reduced cellular lipid accumulation compared with control cells, as assessed by Oil Red O lipid staining and cholesterol quantification. These results suggest that cineole is a selective LXR modulator that regulates the expression of key genes in reverse cholesterol transport in macrophages without inducing lipogenesis in hepatocytes. This selective LXR modulator may have practical implications for the development of hypocholesterolemic or anti-atherosclerotic agents and also suggests.

Cardiovascular diseases are the leading cause of death globally, and it is estimated that almost 23.6 million people will die from cardiovascular disease by the year 2030.[1] Risk of cardiovascular disease has been reported to be associated with cellular cholesterol levels, with excess cellular cholesterol often increasing the risk of cardiovascular diseases such as hypertension and stroke. Therefore, proper control of cholesterol homeostasis is essential. This could be achieved by regulation of liver X receptors (LXRs), which play a significant role in reverse cholesterol transport (RCT), in which accumulated cholesterol is transported from peripheral tissues to the liver, subjected to biliary secretion, and ultimately excreted from the body.[2]

LXRs are ligand-activated transcription factors that belong to the nuclear receptor superfamily. There are two LXR isotypes, LXR-α and -β, which upon activation form heterodimers with the retinoid X receptor and bind to LXR response elements located in the promoter regions of target genes. LXRs have been demonstrated to function as cholesterol sensors and regulators of a set of genes associated with cholesterol absorption, transport, efflux and excretion, and to thus control whole-body cholesterol homeostasis. Considering the importance of LXR activation, there has been increasing interest in the ability of LXR agonists to reduce cellular cholesterol levels and/or risk of cardiovascular disease. Although LXR agonists have shown promising therapeutic effects, various synthetic LXR agonists have been reported to stimulate lipogenesis, leading to hepatic steatosis and hypertriglyceridemia.[3] A previous in vivo study demonstrated that administration of the synthetic LXR agonist T0901317 to mice significantly increased plasma triglyceride levels as a result of activation of sterol regulatory element-binding protein-1c (SREBP-1c).[4] SREBP-1c plays a major role in fatty acid biosynthesis by activating the transcription of various genes that are important in fatty acid synthesis, including the genes encoding acetyl CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl CoA desaturase-1 (SCD-1).[5] Discovery of compounds that act as LXR agonists without inducing lipogenesis is therefore crucial.

1,8-Cineole (cineole), also known as eucalyptol or cajeputol, is a terpene oxide and a principal constituent of most Eucalyptus oils, rosemary, Psidium and many other essential oils.[6] It is often used in the pharmaceutical industry in drug formulations and has been reported to exert various therapeutic effects. The beneficial effects of cineole include analgesic effects, reduced inflammation, and beneficial effects in the treatment of bronchitis, sinusitis and rheumatism.[7] Cineole has also been reported to exert cardiovascular effects, and its consumption significantly reduced the mean aortic pressure and heart rate in mice.[8] However, the mechanisms behind the cardiovascular effects of cineole are not well understood. In addition, no study to-date has evaluated the effect of this natural compound on cardiovascular risk through reduction of cellular cholesterol levels. The potential application of cineole as a novel

* Corresponding author. Tel./fax: +82 2 3290 3494.
E-mail address: junelee@korea.ac.kr (S.-J. Lee).
† These two authors contributed equally to this work.

0960-894X/$ - see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmcl.2012.11.012

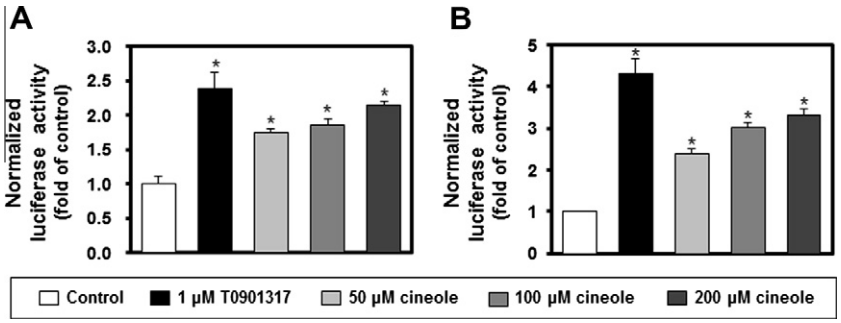

Figure 1. Cineole induced the transactivation of both LXR-α (A) and LXR-β (B). Cells were co-transfected with the reporter vector pGL4.35[luc2P/9XGAL4UAS/Hygro], pSV-β-galactosidase and either PFN26AhLXRα or pFN26AhLXRβ. Luciferase activity was assayed and normalized to that of β-galactosidase.[10] The data shown represent the means ± SE (n = 3); *P <0.05 versus the control.

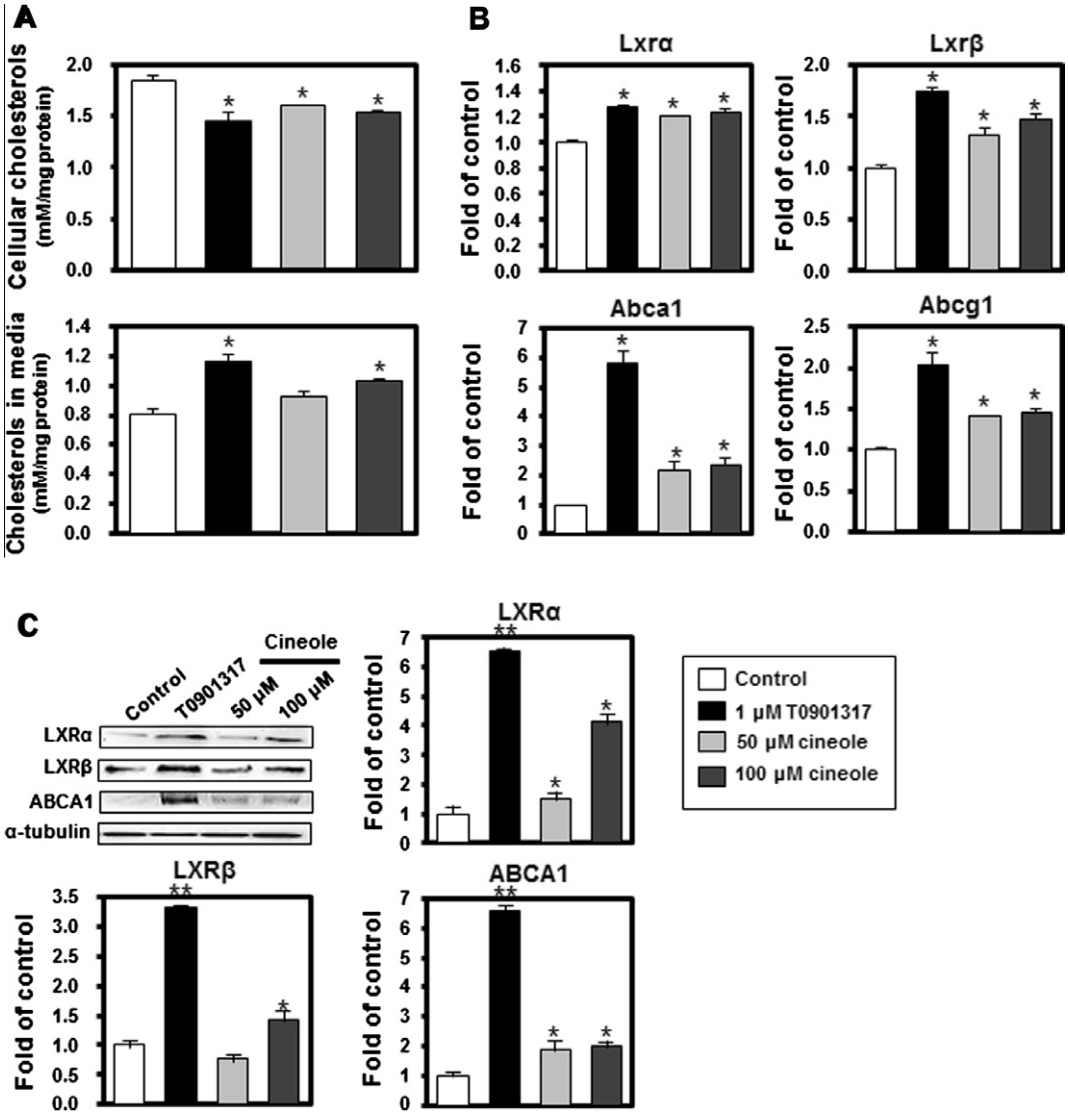

Figure 2. Cineole induced the efflux of cholesterol from RAW 246.7 macrophages by upregulating the gene and protein expression of LXRs and their target genes. (A) Intracellular and extracellular RAW 246.7 cell cholesterol concentrations. (B) mRNA expression of LXR-α, LXR-β, ABCA1 and ABCG1 in RAW 246.7 cells, as measured by qPCR.[14] (C) LXR-α, LXR-β and ABCA1 protein levels in RAW 246.7 cells, as measured by immunoblotting.[16] The data shown represent the means ± SE (n = 3); *P <0.05 and **P <0.01 versus the control.

LXR activator to reduce cholesterol levels warrants further investigation. Therefore, we evaluated the potential effect of cineole on cholesterol efflux and hepatic steatosis in cultured RAW 246.7 macrophages and HepG2 hepatocytes.[9]

In the present study, cineole significantly increased the transactivation of both LXR-α and LXR-β. The effect of cineole on LXR transactivation was assessed by performing luciferase reporter assays in CHO-K1 cells co-transfected with LXRE-Luc reporter vector

and an LXR-α or LXR-β expression vector.[10] Treatment with cineole at 50, 100 and 200 μM induced the transactivation of LXR-α by more than 75% compared to the control ($P < 0.05$) (Fig. 1A). The transactivation of LXR-β was significantly and dose-dependently increased by cineole ($P < 0.05$; Fig. 1B). Transactivation of LXR-β was increased 1.4-, 2.0- and 2.3-fold in cells treated with 50, 100 and 200 μM cineole, respectively.

The activation of LXRs promotes cholesterol efflux, stimulates RCT in macrophages, and inhibits the accumulation of cholesterol in vitro and in vivo.[11] Therefore, we evaluated the cellular cholesterol concentration in lipid-loaded macrophages stimulated with cineole.[12] Cineole, especially at a higher dose (100 μM) significantly reduced the cellular cholesterol level in RAW 264.7 macrophages by 17% compared to control cells ($P < 0.05$) (Fig. 2A). This was accompanied by an increased concentration of cholesterol in the culture medium treated with 100 μM cineole ($P < 0.05$; 35% higher than controls). This suggests that cineole promoted the transport of cholesterol from cells to the medium, thereby reducing cholesterol accumulation in cells and potentially reducing the risk of hypercholesterolemia and atherosclerosis.

It is well-known that LXRs play a critical role in cholesterol homeostasis by inducing the expression of several genes involved in cholesterol efflux, including ABCA1 and ABCG1.[11] ABCA1 regulate the export of cholesterol to lipid free lipoprotein and subsequently generates nascent or pre-β-HDL.[11] These particles in turn serve as substrates for ABCG1-mediated cholesterol transport. Taking into consideration our observation that cineole induced the cholesterol efflux, we further performed quantitative PCR (qPCR) and immunoblotting analysis of LXR target genes and proteins in macrophages, respectively.[12–15] We demonstrated that cineole induced the expression of LXR-α and LXR-β mRNA, similar to T0901317 (Fig. 2B). Treatment with cineole (50 or 100 μM) increased the mRNA expression of LXR-α in macrophages by 20–25% ($P < 0.05$). The mRNA expression of LXR-β was upregulated in a dose-dependent manner, being increased by 35 and 50% in cells treated with 50 and 100 μM cineole, respectively. Cineole also significantly increased the mRNA expression of the LXR-responsive genes ABCA1 and ABCG1 in macrophages ($P < 0.05$). In tandem with the upregulation of mRNA expression, the levels of LXR-α, LXR-β and ABCA1 proteins were also induced in macrophages stimulated with cineole, particularly by 100 μM cineole (Fig. 2C).

Activation of LXRs, leading to macrophage cholesterol efflux, is beneficial and may offer a practical approach for treatment of atherosclerosis. However, the role of LXRs, especially LXR-α, in elevating hepatic fatty acid synthesis served as a potential side-effect of LXR therapy.[3] Nakamuta et al.[16] and others demonstrated that activation of LXR-α caused hepatic steatosis and hyperlipidemia in human subjects through the induction of SREBP-1c. SREBP-1c is a key factor involved in regulating the expression of several hepatic lipogenic enzymes, including acetyl coenzyme A carboxylase,

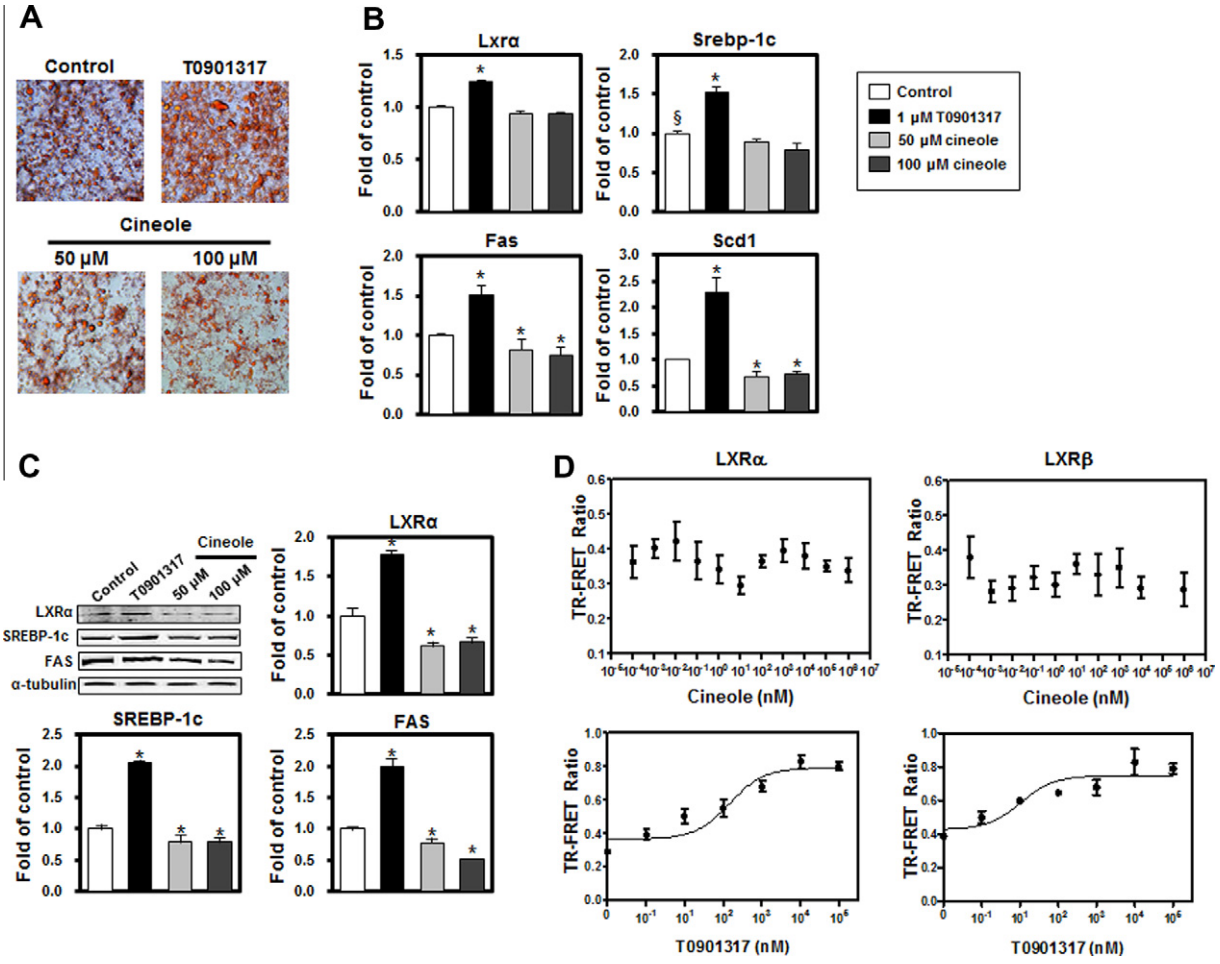

Figure 3. Cineole reduced hepatic lipid accumulation by downregulating the gene and protein expression of LXR-α and its target genes in hepatocytes. (A) Lipid accumulation in HepG2 cells, as assessed by Oil Red O staining.[18] (B) mRNA expression of LXR-α, SREBP-1c, FAS and SCD1 in HepG2 cells, as measured by qPCR.[13] (C) LXR-α, SREBP-1c and FAS protein levels in HepG2 cells, as measured by immunoblotting.[15] (D) LanthaScreen TR-FRET assays were performed interactions between cineole and LXR-α and LXR-β.[21] T090137 was used as a positive control. The data shown represent the mean ± SE ($n = 3$); *$P < 0.05$ versus the control.

fatty acid synthase (FAS) and stearoyl coenzyme A desaturase-1 (SCD-1).[17] Thus, in theory, the most successful strategies would involve macrophage-specific upregulation of LXRs without inducing hepatic lipogenesis.

In this study, treatment with cineole led to a decrease in cellular lipid accumulation in hepatocytes treated with cineole (50 or 100 μM), as assessed by Oil Red O staining (Fig. 3A).[18] On the other hand, T0901317-treated cells showed significant accumulation of cellular lipid droplets. This effect of cineole was due to unaltered expression of LXR-α and downregulation of the expression of SREBP-1c, FAS and SCD-1 by 10%, 25%, 24% and 30%, respectively (Fig. 3B). In addition, cineole (100 μM) substantially reduced the protein expression of LXR-α (by 40%), SREBP-1c (25%) and FAS (50%) in hepatocytes ($P < 0.05$; Fig. 3C). This indicates that, unlike T0901317, cineole acts as a partial agonist that selectively activates LXRs without inducing hepatic lipogenesis. Thus, treatment with this compound may have practical implications for the prevention or treatment of dyslipidemia without undesirable side effects.

The mechanisms of cineole in the downregulation of LXRs, SREBP1c, and FAS in hepatocytes are currently unclear. However, there are three possibilities. First, it has been shown that ligand compounds could recruit co-activators/co-repressors differentially, which could result in tissue-specific activity. Albers et al.[19] compared the effects of T0901317 with those of LN6500 in the induction of two LXR target genes, FAS and SCD-1 in the liver. Regarding LXR-α, T0901317 potently induced the expressions of FAS and SCD-1 while LN6500 showed no induction. Interestingly, in co-activator recruitment assay, T0901317 recruited TRAP220 strongly, while LN6500 only recruited TRAP220 weakly, which may explain no induction of FAS and SCD-1 expression by LN6500. To evaluate the possibility of direct interactions between cineole and LXR-α and LXR-β, a cell-free FRET co-activator assay was performed with LXR co-activator peptides and the LBDs of LXR-α and LXR-β.[20] As expected, the potent LXR agonist T0901317 showed strong binding to both LXR-α (EC_{50} = 0.32 μM) and LXR-β (EC_{50} = 0.01 μM), and induced the recruitment of Trap 220 and D22 co-activator peptide to the LXR-α and LXR-β LBDs, respectively, in a dose-dependent manner (Fig. 3D). Cineole recruits the TRAP220/DRIP-2 and D22 coactivators very weakly to LXR-α and LXR-β, respectively. Hence, it is possible that LXR-α-activation by cineole may recruit co-activators other than TRAP220 thus showed slightly different metabolic effects compared with those by T0901317. Alternatively, it is also possible that cineole may not directly activate LXR proteins but indirectly by inducing endogenous ligand synthesis.

Second, cineole may have additional effects on the cellular lipid metabolism. It has been reported that AMPK activation reduces hepatic but not macrophage LXRα.[21,22] Although not investigated in the present study, it possible that the net expression of hepatic LXR, SREBP1c, and FAS is controlled by the combined effects of LXR and AMPK activity modulated by cineole.

Third, some natural compounds have shown that posttranscriptional modification of SREBP-1 could be delayed by inducing the expression of Insig-2a, thus inhibiting nuclear translocation of pSREBP to nSREBP in hepatocytes.[16,23,24] Cineole may have similar activities.

In conclusion, cineole acts as a selective LXR modulator that activates the expression of LXR genes in a tissue-specific manner. In macrophages, cineole significantly activated the expression of LXRs and a spectrum of genes that regulate reverse cholesterol transport. In contrast, it downregulated the expression of LXR-α and lipogenesis-related genes in the liver. Cineole can serve as a potent pharmaceutical agent for treatment of hypercholesterolemia and atherosclerosis since it not only reduces cholesterol accumulation, thereby preventing atherosclerotic plaque formation, but also prevents the potential side effect hepatic steatosis.

Acknowledgments

We appreciate the technical assistance of Hea-Won Kim. This study was supported by the Korean Forest Service (Forest Science & Technology Project No. S120912L020110), by "Cooperative Research Program for Agricultural Science and Technology Development (PJ00842203, No. 20120301030609001040)", Rural Development Administration, Republic of Korea, and by the Basic Science Research Program through the National Research Foundation of Korea (NRF, 20110027386).

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9. Murine macrophage-like RAW 264.7 cells and HepG2 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS and 1% PES before treatment. All cell lines were grown in 5% CO_2 at 37 °C. For experiments, RAW 264.7 macrophages and HepG2 cells were preincubated with DMEM for 24 h. The following day, the medium was removed and the cells were incubated for an additional 24 h in 2 mL of medium containing 1 μM T0901317 or cineole (50 or 100 μM). Stock solutions of cineole (10 mM; Sigma, St. Louis, MO, USA) and T0901317 (1 mM; Sigma) were prepared in DMSO. Vehicle control cells were treated with a concentration of DMSO (1%) corresponding to that used for the highest substance concentration. Each treatment was applied in at least triplicate.
10. The CHO-K1 cells used in luciferase reporter assays were maintained in Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM/F12) medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (PES). Initially, CHO-K1 cells were seeded in 24-well plates (1×10^5 cells per well). The following day, the cells were co-transfected with the reporter vector pGL4.35[luc2P/9XGAL4UAS/Hygro], pSV-β-galactosidase and either PFN26AhLXRα or pFN26AhLXRβ using Hilymax transfection reagent (Dojindo Laboratories, Rockville, MD, USA). After 24 h, the cells were treated with cineole (50, 100 or 200 μM) or T0901317 (1 μM) for 24 h and lysed in Firefly Luciferase Lysis Buffer (Biotium, Inc., Hayward, CA, USA). The luciferase activity in the cell lysate was measured using a Firefly Luciferase Assay Kit (Biotium), according to the manufacturer's protocol. β-Galactosidase activity was determined using the β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA). To normalize the results for transfection efficiency, luciferase activity was expressed relative to the β-galactosidase activity in the same lysate.
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12. Cells were washed with ice-cold PBS and cellular lipids were extracted at room temperature with 2 mL of a 2:1 (v/v) mixture of hexane and isopropanol. The organic solvent was removed by vacuum centrifugation and the lipids were resuspended in 200 μL of 95% ethanol. Cholesterol levels were measured using an Amplex Red Cholesterol Assay Kit (Invitrogen) according to the manufacturer's instructions.
13. Total RNA was extracted from RAW 264.7 macrophages and HepG2 cells using an RNAiso Plus kit (Takara, Otsu, Japan) according to the manufacturer's protocol after treatment for 1 day with T0901317, cineole or vehicle (1% DMSO). Real-time qPCR was performed with Bio-Rad iQ SYBR→ Green Supermix reagent (Takara, Otsu, Japan) and the Bio-Rad iQ5 Cycler System. The primers used are described in Hoang et al.[14] Expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase or cyclophilin by the normalized expression (CT) method according to the manufacturer's guidelines.
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15. RAW 264.7 macrophages and HepG2 cells were lysed in ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.4), 0.1 M EDTA, 10 mM NaCl, 0.5% Triton X-100, and protease inhibitor cocktail (Roche, Mannheim, Germany). The lysate was clarified by centrifugation at 14,000 rpm for 10 min at 4 °C. Protein concentration was determined using a Bio-Rad protein kit with bovine serum albumin (Sigma) as the standard. Protein samples (30 μg) were subjected to 10% SDS–PAGE and then transferred and immobilized on nitrocellulose

membranes. After blocking, the membranes were probed with primary antibody (anti-LXR-α, anti-LXR-β, anti-ABCA1, antiABCG1, anti-SREBP 1, anti-FAS or anti-α-tubulin; Santa Cruz Biotechnology) and then incubated with secondary antibody (anti-rabbit or anti-mouse immunoglobulin G; Santa Cruz Biotechnology). Immunoreactive bands were imaged with a ChemiDoc XRS imaging system (Bio-Rad) using PowerOpti-ECL Western blotting detection reagent (Anigen). Relative band intensities were determined using Gel-Pro Analyzer 4.0 software (Media Cybernetics). For each sample, target protein levels were normalized to α-tubulin (internal reference).

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18. HepG2 cells were washed with ice-cold PBS and fixed overnight with formalin (10% v/v). Next, fixed cells were washed with water and isopropanol (60% v/v), and stained with Oil Red O (0.35% v/v) for 1 h. After washing with water, microscopic images were captured.
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