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Ethyl 2,4,6-trihydroxybenzoate is an agonistic ligand for liver X receptor that induces cholesterol efflux from macrophages without affecting lipid accumulation in HepG2 cells

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ABSTRACT

The present study reports a novel liver X receptor (LXR) activator, ethyl 2,4,6-trihydroxybenzoate (ETB), isolated from *Celtis biondii*. Using a reporter gene assay, time-resolved fluorescence resonance energy transfer (TR-FRET), and surface plasmon resonance (SPR) analysis, we showed that ETB directly bound to and stimulated the transcriptional activity of LXR-α and LXR-β. In macrophages, hepatocytes, and intestinal cells, ETB suppressed cellular cholesterol accumulation in a dose-dependent manner and induced the transcriptional activation of LXR-α/-β-responsive genes. Notably, ETB did not induce lipogenic gene expression or cellular triglyceride accumulation in hepatocytes. These results suggest that ETB is a dual-LXR modulator that regulates the expression of key genes in cholesterol homeostasis in multiple cells without inducing lipid accumulation in HepG2 cells.

Atherosclerosis is the leading cause of death worldwide, accounting for approximately 72 million deaths each year.[1] Epidemiological studies have identified high levels of low-density lipoprotein (LDL) cholesterol and reduced levels of high-density lipoprotein (HDL) cholesterol as major contributors to atherogenesis.[2] Accordingly, HDL-raising therapies have generated interest. It was recently demonstrated that nuclear receptor LXRs regulate the cholesterol and phospholipid pump by activating the transcription of ATP-binding cassette (ABC) A1/G1/G5/G8 and apolipoprotein E (apoE). Thus, LXR agonists are potential HDL-raising agents that could act on the artery wall to stimulate the efflux of cholesterol from lipid-laden macrophages. Treatment of atherosclerotic mice with synthetic LXR ligands, such as GW3965 and T0901317, inhibit progression and promote regression of atherosclerotic plaques. Furthermore, transplantation of macrophages lacking LXR-α/-β into a host that is predisposed to atherogenesis results in increased foam cell differentiation and arterial plaque formation, even after treatment with LXR agonists.[3,4]

These results highlight the cardioprotective roles of LXRs. However, studies have also shown that LXR agonists can cause liver steatosis and increase serum triglyceride levels in rodents by activating hepatic SREBP-1c.[5,6] Thus, specific LXR ligands that do not induce fatty acid synthesis in the liver are of interest. Several groups have described agents that have beneficial effects on lipid metabolism. Vlasuk et al.[7] and Kratzer and co-workers[8] identified two novel LXR agonists, WAY-252623 and *N,N*-dimethyl-3β-hydroxy-cholenamide, that reduce atherosclerosis without activating SREBP1c or increasing hepatic lipogenesis. This raised the possibility that some of the anti-atherosclerotic effects of LXR agonists may be independent of systemic lipid metabolism in hepatocytes and may be attributable to direct actions on the vascular wall that activate reverse cholesterol transport (RCT). Hence, LXR is an attractive target for novel pharmaceutical agents.

Plant medicines have become increasingly popular for the prevention and/or treatment of cardiovascular disease and have proven to be an abundant source of pharmacological agents for medicinal purposes. The characterization of key active compounds is essential.[9] Initially, we screened approximately 900 Korean medicinal plant and lipidarine extracts for LXR agonist activity and found that *Celtis biondii* (*CB*) ethanol extract had potent LXR agonist activity. In this study, we isolated an LXR agonistic

Abbreviations: ABC, ATP-binding cassette transporter; FAS, fatty acid synthase; HDL, high-density lipoprotein; LBD, ligand-binding domain; LDL, low-density lipoprotein; LXR, liver X receptor; NPC1L1, Niemann-Pick C1 Like 1; SCD-1, stearoyl-CoA desaturase 1; SPR, surface plasmon resonance; SREBP, sterol regulatory element-binding protein; TG, triglyceride; TR-FRET, timeresolved fluorescence resonance energy transfer.

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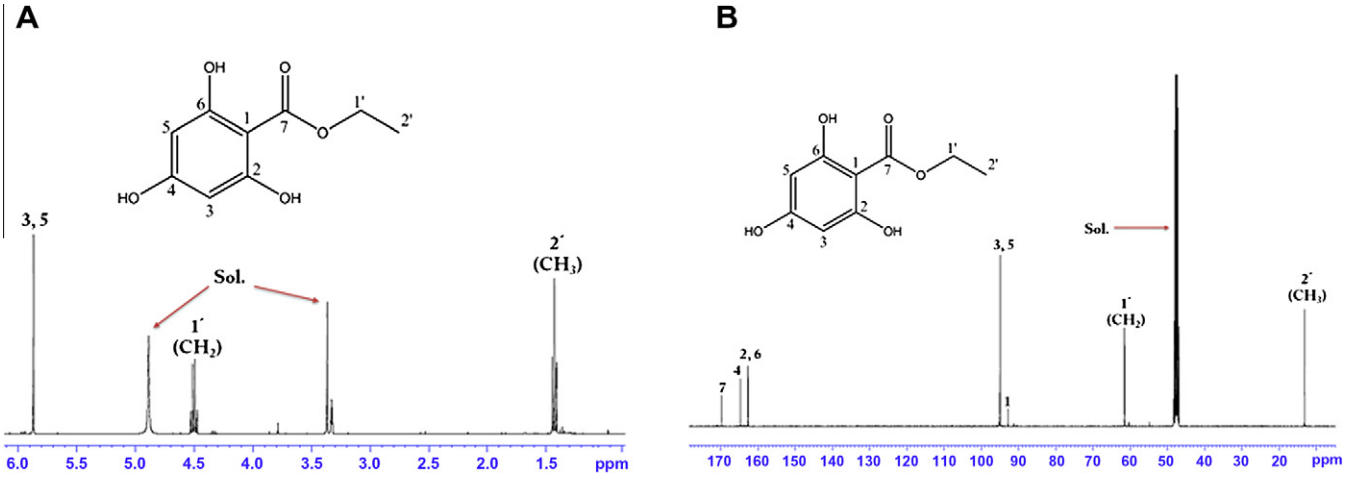

Figure 1. ^{1}H NMR (A) and ^{13}C NMR (B) spectra for ETB.

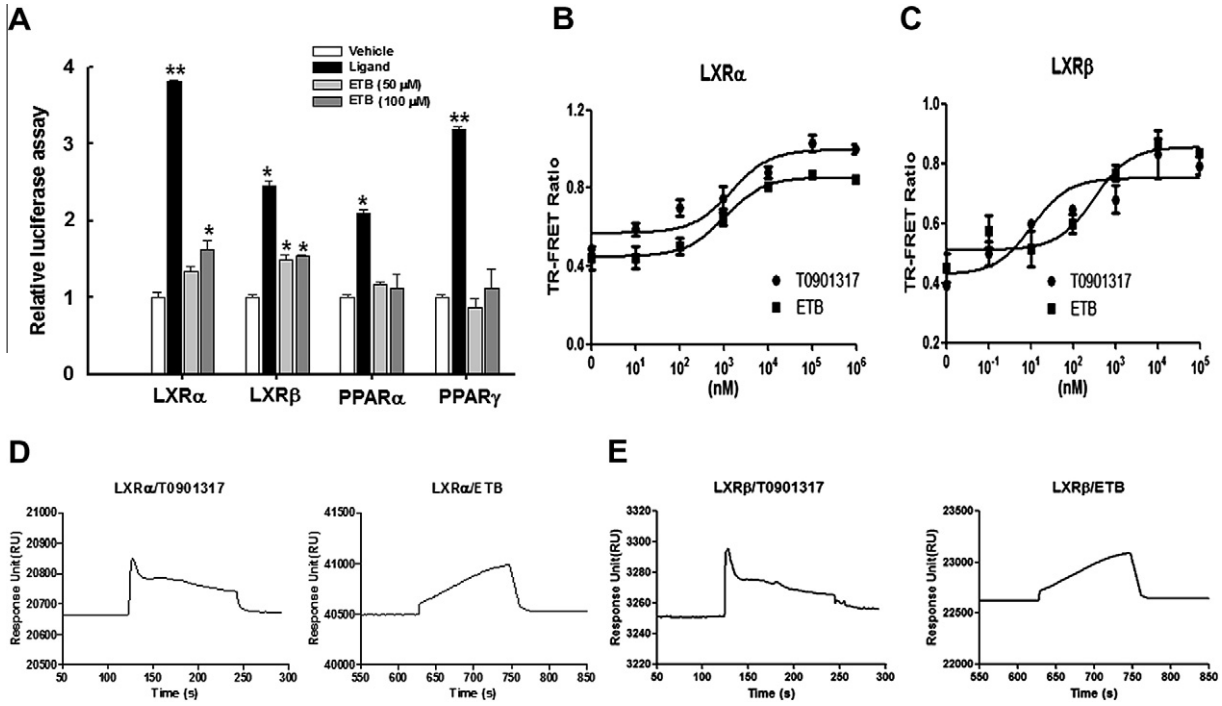

Figure 2. ETB activates LXR-α and LXR-β by directly interacting with their ligand-binding domains. (A) ETB activates LXR-α and LXR-β but not PPAR-α and PPAR-γ transactivation activity. HEK 293 cells were transfected with expression plasmids for receptors and a pSV-β-galactosidase together with a reporter plasmid (pGL4.35[luc2P/9XGAL4UAS/Hygro] for LXRs and pCMV-3xPPRE-Luc for PPARs). The cells were exposed to ETB (50 and 100 μM), receptor-specific ligands (1 μM T0901317 for LXRs, 10 μM fenofibric acid for PPAR-α, and 10 μM troglitazone for PPAR-γ), or vehicle control (1% DMSO) for 24 h before assaying luciferase activity. Activation of LXR-α (B) and LXR-β (C) by T0901317 and ETB as assessed by TR-FRET assay. The direct interactions between ETB (100 μM) or T0901317 (1 μM) and the immobilized LXR-α-LBD (D) and LXR-β-LBD (E) were examined by SPR on a Biacore instrument. Data represent the mean ± SE; $^{*}P$ <0.05 versus control (n = 8).

compound from *CB* ethanol extract and investigated its metabolic effect on lipid metabolism.

Lyophilized *CB* root powder was extracted with EtOH at room temperature for 72 h. The EtOH extract (81.23 g) was partitioned into two subfractions using organic solvents, yielding *n*-hexane-soluble (4.26 g) and CH_2Cl_2-soluble (4.14 g). The CH_2Cl_2 fraction was further subjected to multiple chromatographic steps on a Sephadex LH-20 column (6.0 × 47.0 cm) to provide the compound CBD-III (Supplementary Fig. 1).

Next, the structure of this compound was elucidated using MS, ^{1}H, and ^{13}C NMR spectra analysis[10] and by direct comparison with published data.[11] The compound produced a molecular ion peak at 188 $[M]^{+}$ in the EI-MS spectrum. The ^{1}H NMR spectrum showed ethyl signals at δ 1.43 (3H, t, H-2′) and δ 4.50 (2H, *dd*, H-1′) and an aromatic proton signal at δ 5.87 (2H, *s*, H-3, H-5). The low-field shift of the methylene group was suggestive of an ester group (Fig. 1A). In the ^{13}C NMR spectrum, there were two ethyl group signals [δ 13.22 (*q*, C-2′) and δ 61.59 (*t*, C-1′)], four aromatic signals [δ 92.86 (*s*, C-1), 162.66 (*s*, C-2, C-6), 164.73 (*s*, C-4)], and an ester carbonyl signal [δ 169.76 (*s*, C-7)]. Among the aromatic C signals, the chemical shifts at δ 95.00 (*d*, C-3, C-5) and δ 92.86 (*s*, C-1) were attributable to the C–H and C–C groups, respectively, and the remaining signals at δ 164.73 (*s*, C-4) and δ 162.66 (*s*, C-2, C-6) were attributable to a C–O group, indicative of a methyl 2,4,6-trihydroxybenzoate moiety (Fig. 1B).[11] Based on these data, the compound was identified as ethyl 2,4,6-trihydroxybenzoate (ETB).