Bioorganic & Medicinal Chemistry Letters 23 (2013) 4185–4190

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Cyanidin, a natural flavonoid, is an agonistic ligand for liver X receptor alpha and beta and reduces cellular lipid accumulation in macrophages and hepatocytes

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ARTICLE INFO

Article history:
Received 16 November 2012
Revised 27 April 2013
Accepted 9 May 2013
Available online 18 May 2013

Keywords:
Cyanidin
LXR
Ligand
Macrophages
Hepatocyte

ABSTRACT

Cyanidin, a natural flavonoid abundant in fruits and vegetables, is known to regulate cellular lipid metabolism; however, its underlying mechanism of action and protein targets remain unknown. Here, the ligand binding activity of cyanidin on liver X receptors (LXRs) was investigated utilizing surface plasmon resonance and time-resolved fluorescence energy transfer (TR-FRET) analyses. LXRs are nuclear receptors which function as critical transcription factors in the regulation of cellular lipid and glucose metabolism. This includes the stimulation of high-density-lipoprotein synthesis and activation of reverse cholesterol transport. The present findings show that cyanidin induces the transactivation of LXRs and binds directly to the ligand-binding domain of both LXRα and LXRβ with dissociation constants of 2.2 and 73.2 μM, respectively. Cell-free FRET analysis demonstrated that cyanidin induces the recruitment of co-activator peptide for LXRα and LXRβ with EC_{50} of 3.5 μM and 125.2 μM, respectively. In addition, intracellular cholesterol and triglyceride (TG) concentrations were reduced in macrophages following cyanidin stimulation. In cultured hepatocytes, cyanidin mildly induced SREBP1c gene expression but marginally affected cellular TG concentrations as well as reduced cellular cholesterol accumulations which activated the expression of genes for reverse cholesterol transport. Two cyanidin metabolites, procatechic acid and phloroglucinaldehyde, did not directly bind or activate LXRs. These results demonstrate that cyanidin is a direct ligand for both LXRα and LXRβ, suggesting that cyanidin may operate, at least in part, through modulation of cellular LXR activity.

The liver X receptor (LXR) is a nuclear receptor that induces transcription of target genes in cellular cholesterol, fatty acid, and glucose homeostasis.[1,2] Two LXR isoforms, LXRα and LXRβ, have different tissue distributions with LXRβ being expressed ubiquitously while LXRα expression is restricted to metabolically active tissues such as liver, kidney, intestine, fat tissue, and macrophages with the highest level of expression in the liver.[3]

Known ligands for LXRs, such as oxysterols,[4] glucose,[5] and T0901317,[6] bind to both LXR subtypes and form a heterodimer with the obligate partner 9-*cis* retinoic acid receptor (RXR). This heterodimer binds to the hepatic lipogenesis activating sterol regulatory element binding protein 1c (SREBP1c)[7] as well as the LXR response element (LXRE) in the promoter region of target genes important in cholesterol and glucose metabolism.[8]

Considering the importance of LXRs in the physiology of lipid and cholesterol metabolism, agonists for LXRs have been suggested as a potential therapy for metabolic disorders such as hyperlipidemia and atherosclerosis.[9] LXRs function as cholesterol sensors and regulators of the genes associated with cholesterol absorption, transport, efflux, and excretion and in effect regulate whole body cholesterol homeostasis.[10] Therefore, the activation of LXRs may result in improved reverse cholesterol transport and an increase in circulating high-density-lipoprotein (HDL) levels. Several groups have reported a dose-dependent induction of HDL cholesterol concentrations in C57BL/6 mice following administration of the LXR dual agonist, T0901317.[6] In addition, LXRs act as master regulators of hepatic lipid metabolism. Administration of T0901317 to mice significantly lowers serum and hepatic cholesterol concentrations and inhibits the development of atherosclerosis[11,12] However, synthetic LXR agonists may induce lipogenesis which leads to increased plasma triglyceride (TG) concentrations and hepatic steatosis mainly by inducing SREBP1c in the liver.[6] Accordingly, the development of novel LXR agonists that do not induce hepatic steatosis is of great interest for clinical applications.

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0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.05.030

An activity screening experiment using a reporter gene assay identified cyanidin as a potential LXR agonist, which initialized the current study. Cyanidin is an anthocyanin abundant in fruits and vegetables with potent antioxidant and radical-scavenging effects.[13,14] The dietary intake of cyanidin may inhibit the development of dyslipidemia and reduce inflammation.[15] Anthocyanin supplementation including cyanidin in humans decreases the concentrations of low-density lipoprotein (LDL) and increases the concentration of high-density lipoprotein (HDL) cholesterol thus enhancing cellular cholesterol efflux to the serum.[16] The administration of cyanidin-3-*O*-β-glucoside to apoE-deficient mice reduces total serum cholesterol level, increases serum HDL concentration, and ameliorates hypercholesterolemia-induced endothelial dysfunction and atherosclerosis via the ABCG1 pathway.[17] Moreover, the chronic administration of cyanidin-3-glucoside significantly increases HDL-cholesterol whereas significantly declines LDL cholesterol levels without changing total serum cholesterol or TG levels.[18] The administration of red wine phenolic compounds containing cyanidin to hypercholesterolemic Golden Syrian Hamsters significantly reduces plasma cholesterol, TG, and apolipoprotein B concentrations, and decreases the aortic fatty streak area,[18] suggesting that cyanidin may additionally modulate lipid metabolism. Although ample data have shown that cyanidin has hypolipidemic and HDL-enhancing effects, its direct molecular target remains unknown. Here, the direct interaction between cyanidin and the ligand binding domain (LBD) of LXR proteins was investigated in addition to its hypolipidemic effects via regulation of LXR target gene expression.

The transactivation of LXRα and β was induced with stimulation of cyanidin in CHO-K1 cells was assessed with luciferase assay.[19] Cyanidin transactivated LXRα by 32% and 59% at 50 and 100 μM, respectively; while T0901317 (2 μM) induced the promoter activity by 96% compared with controls (Fig. 1B). Cyanidin (100 μM) induced the transactivation activity of LXRβ by 33% whereas T0901317 (2 μM) activated LXRβ promoter by 45% compared with controls (Fig. 1C). The interaction of cyanidin with LXR-LBD was quantified using the surface plasmon resonance (SPR)-BIAcore system[20] and demonstrated that cyanidin directly associated with both LXR subtypes (Fig. 1D and E). The K_D of cyanidin to LXRα and LXRβ was 2.16 and 73.2 μM, respectively (Table 1). The synthetic agonist ligand of the LXRs, T0901317, also directly bound with both LXRα and LXRβ, with a K_D of 92 and 103 nM, respectively. Although the binding affinity of cyanidin to the LXRs was lower compared to T0901317, these results demonstrate that cyanidin directly bound to both subtypes of LXRs with a higher affinity for LXRα than LXRβ.

To further investigate the agonist activity of cyanidin for the two LXR subtypes, LXRα- and LXRβ-LBD proteins were incubated with the co-activator peptide for the corresponding LXR subtype at different concentrations of cyanidin or T0901317 (from 10^{-4}

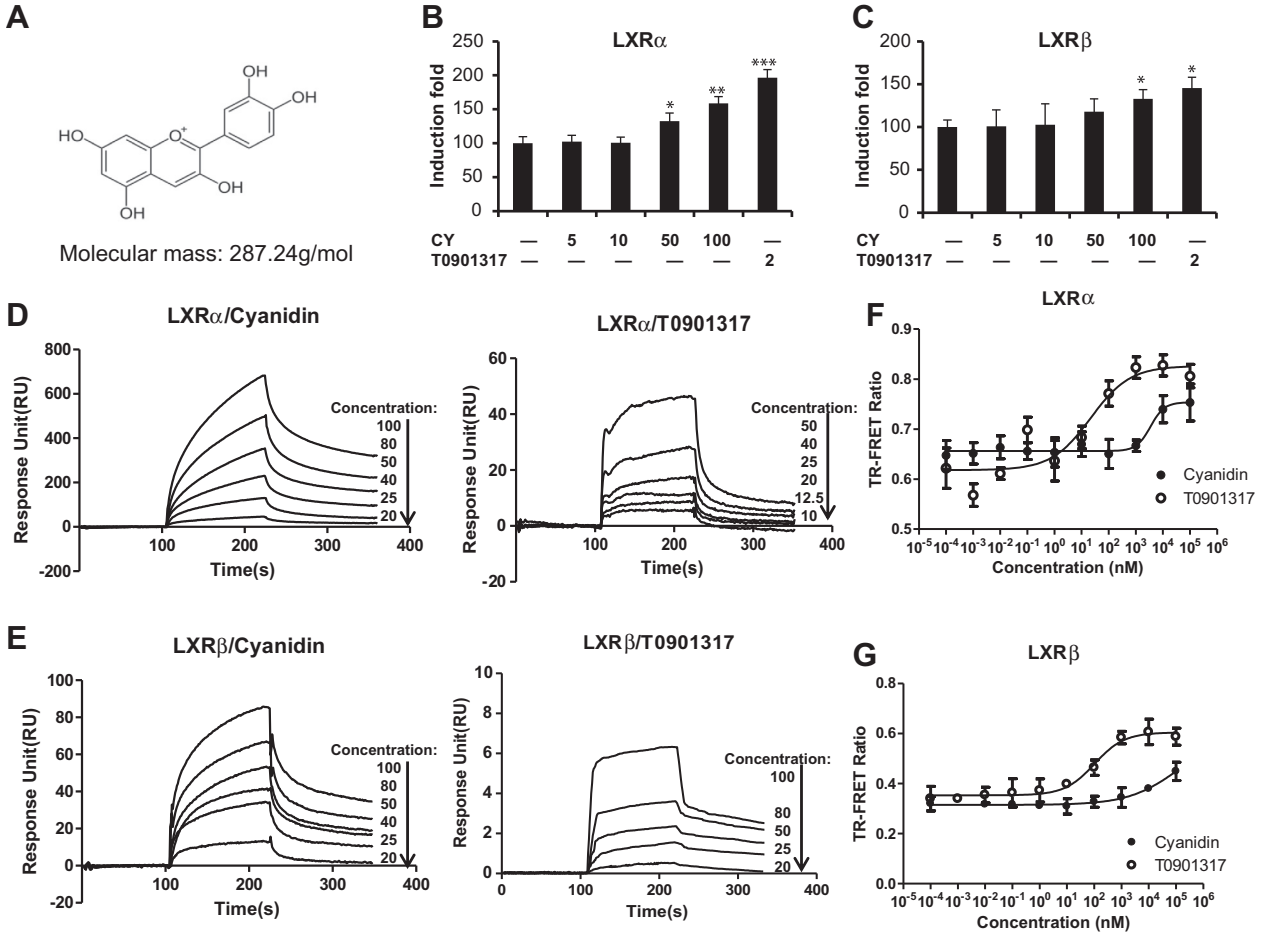

Figure 1. Cyanidin induces transactivation of LXRα and LXRβ, binds directly to LXRα and LXRβ and induces the recruitment of LXR co-activator peptides by activation of LXRα and LXRβ in a TR-FRET assay. (A) Structure of cyanidin. Cyanidin induces transactivation of LXRα (B) and LXRβ (C) measured by luciferase assay. SPR sensorgrams were obtained from Biacore 2000 after injection of a series of concentrations of cyanidin (CY, left panel of B and C), T0901317 (right panel of D and E) over the immobilized hLXRα-LBD or hLXRβ-LBD response. Cyanidin and T0901317 were incubated with LXRα-LBD (F) and LXRβ-LBD (G). $^{*}P$ <0.05, $^{**}P$ <0.001 and $^{***}P$ <0.001 compared to controls. Concentrations are shown in μM.

Table 1
Equilibrium dissociation constants (K_D) of cyanidin and synthetic LXR agonist T0901317 to hLXRα-LBD and hLXRβ-LBD quantified by SPR

Compound	Equilibrium dissociation constants (K_Ds)	
	hLXRα-LBD	hLXRβ-LBD
Cyanidin	2.2 μM	73.2 μM
T0901317	92.4 nM	103.0 nM

Table 2
Half-maximal effective concentration (EC_{50}) of cyanidin and synthetic LXR agonist T0901317 to hLXRα-LBD and hLXRβ-LBD quantified by TR-FRET assay

Compound	Half maximal effective concentration (EC_{50})	
	hLXRα-LBD	hLXRβ-LBD
Cyanidin	3.5 μM	125.2 μM
T0901317	91.1 nM	102.6 nM

to 10^5 nM), and analyzed using time-resolved fluorescence resonance energy transfer (TR-FRET) co-activator assays[21] (Fig. 1F and G). Cyanidin activated LXRα (Fig. 1F) with a half-maximal effective concentration (EC_{50}) value of 3.48 μM (Table. 2) and induced co-activator activity of LXRβ with a EC_{50} value of 125.2 μM (Fig. 1G). The activity of T0901317 relative to the LXRs (Fig. 1F and G) showed an EC_{50} of 91 nM at LXRα, and 102 nM at LXRβ. This demonstrates that cyanidin is an agonist for the LXRs with a larger effect at LXRα.

The EC_{50} (3.5 μM) of cyanidin is similar to those of endogenous oxysterol ligands. The most potent oxysterol, 22*R*-hydroxycholesterol, shows an EC_{50} of ~1 μM for LXRα. Cyanidin and endogenous ligands will compete for the binding domain of LXR when both ligands are present. Cyanidin and oxysterols may show similar LXR activation when both are present because they have a similar EC_{50}. Cyanidin is a relatively non-toxic natural compound, and a large volume of published data has demonstrated its bioactivity without affecting cell viability at high concentrations (e.g., 100 μM).

The assay results indicate that cyanidin directly binds to and activates both LXR subtypes, which agrees with the SPR results. Anti-atherogenic activities of LXRα and LXRβ agonists have been confirmed in mice.[9,22] Therefore, it is possible that cyanidin exerts a similar beneficial effect in vivo to those exerted by known LXR agonists; this potential benefit should be confirmed in future studies. Although the activity in cyanidin is much lower than that elicited by the synthetic agonist, T0901317, an adequate intake of fruits and vegetables, especially berries, could provide sufficient plasma cyanidin concentrations capable of activating LXR-related metabolic effects.

The effects of cyanidin on the LXR subtypes were slightly different, as evidenced by the SPR and TR-FRET co-activator assays. At present, we cannot fully explain these findings; however, it is reasonable for a ligand to interact with several protein subtypes with differing binding affinities. In fact, the ligand-binding pocket of LXR isoforms can adopt various shapes and volumes to accommodate a diverse array of ligands of varying size and structure.[23,24] It has been suggested that the interaction of the hydroxyl/epoxide moieties of an LXR ligand with His-421 (LXRα) and His-435 (LXRβ) is the most essential point of contact in LXR and agonist complexes.[23–25] In silico ligand docking results revealed that gynosaponin TR1 isolated from *Gynostemma pentaphyllum*, a selective agonist for LXRα, forms a hydrogen bond with His-421 of LXRα but not with His-435 of LXRβ.[26] Similarly, cyanidin may interact differentially to LXR subtypes due to a different orientation and distance from the key amino acid residues in the binding pockets of the two LXRs.

On the other hand, pharmacophore modeling of LXRβ-specific three dimensional pharmacophores has suggested that a selective agonist of LXRβ should contain one hydrogen acceptor and four hydrophobic features.[27] Cyanidin is water soluble and hydrophilic and does not satisfy the criteria for the LXRβ-specific ligand, which was confirmed by our data.

Procatechic acid (PCA) and phloroglucinaldehyde (PGA) do not interact with LXR-LBDs. Cyanidin and its glycosides can be degraded and metabolized into PCA and PGA. Thus, we evaluated whether PCA or PGA could directly interact with LXR-LBDs. In both SPR analyses and TR-FRET assays, neither PCA nor PGA significantly bound to LXR-LBDs (Fig. 2A) and neither recruited a co-activator peptide to the LXR-LBD (Fig. 2B). These data confirmed that cyanidin metabolites do not directly bind to LXRs.

Cyanidin induces the transcription of LXRα, LXRβ, and LXR responsive gene expression. The expression of responsive genes for LXR and LXRβ were quantified in hepatocytes stimulated with cyanidin.[28] Total RNA was extracted from HepG2 cells and real-time qPCR was performed as described previously.[29,30] Cyanidin activated LXR transcription by 1.5-fold at both 50 μM and 100 μM

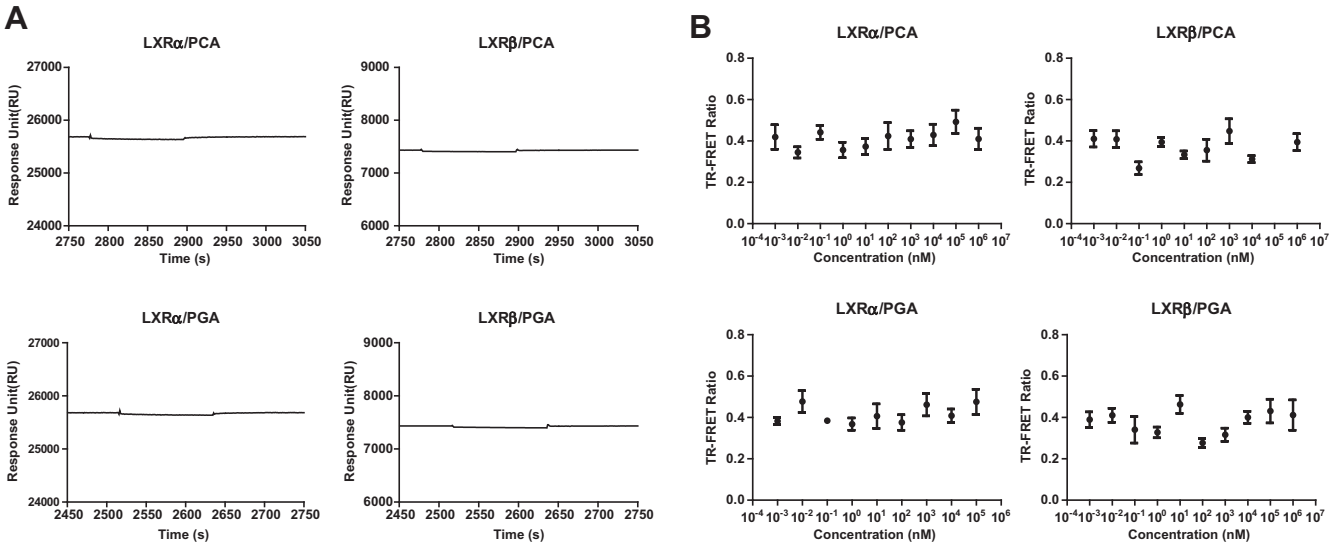

Figure 2. PGA and PCA do not bind to LXR subtypes in SPR and cell-free FRET assays. (A) Sensorgrams were obtained by injection of PCA or PCA (100 μM each) over the immobilized hLXRα-LBD or hLXRβ-LBD response; (B) TR-FRET ratio of PCA and PCA for LXR co-activator recruitment.

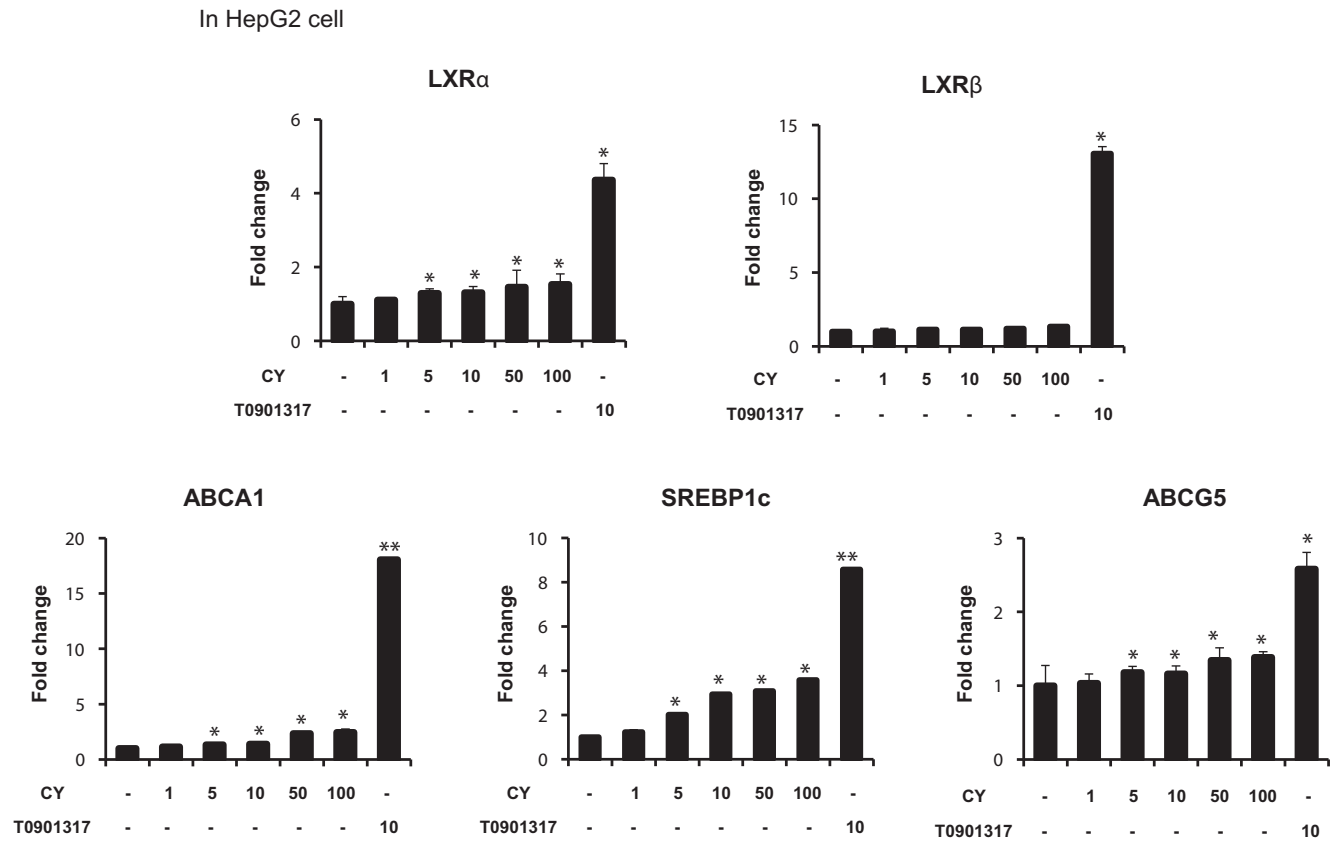

Figure 3. Cyanidin regulates the expression of LXRs and their specific target genes. HepG2 cells were stimulated with cyanidin or T0901317, and vehicle DMSO (1%) for 24 h and changes in gene expressions were assessed by qPCR. Results are normalized to the GAPDH mRNA level. $^{*}P$ <0.05 and $^{**}P$ <0.001 compared to controls. Concentrations are shown in μM.

but did not alter LXRβ expression (Fig. 3). The gene expression of LXR but not LXRβ is self-regulated,[31] thus, these data support the known effects of ligand-dependent LXR activation. Cyanidin also activated LXR responsive genes including ABCA1, SREBP1c, and ABCG5 by 2.5-fold (100 μM), 3.6-fold (100 μM), and 1.4-fold (100 μM), respectively, while the synthetic LXR agonist T0901317 (10 μM) up-regulated ABCA1, SREBP1c, and ABCG5 by 18.1-fold, 8.6-fold, and 2.6-fold, respectively. The results were in line with the results from the SPR and cell-free FRET assays of cyanidin to LXR and LXRβ.

LXR activation reduces cellular cholesterol concentrations via multiple mechanisms including the stimulation of cholesterol removal, reverse cholesterol transport, the inhibition of intestinal cholesterol absorption, cholesterol synthesis and uptake, and induction of biliary excretion.[32] Numerous in vitro[33] and in vivo[34] studies have shown that ABCA1, which mediates cholesterol efflux from macrophages and hepatocytes, is a definite LXR target gene. ABCA1 is a full transporter that transfers both cholesterol and phospholipids from plasma membranes to small discoid-shape pre-HDL forms or to lipid-free apoA-I which contributes to the formation of nascent HDL particles in liver and small intestine.[34] The induction of cholesterol efflux by LXR ligands is ABCA1-dependent.[35] Thus, cyanidin stimulation of ABCA1 induction may improve cholesterol efflux and increase HDL concentration by promoting reverse cholesterol transport.

A substantial amount of intracellular cholesterols are also effluxed through ABCG5/ABCG8 heterodimers, particularly in hepatocytes, macrophages, and the intestinal epithelium.[36] ABCG5/ABCG8 is a cholesterol transporter highly expressed in the intestinal epithelium that mediates cholesterol efflux.[37] ABCG5 and ABCG8 are also abundantly expressed in the canalicular membrane of hepatocytes and aid in the transport of cholesterols into the bile duct. Hepatic ABCG5 and ABCG8 expressions are stimulated by LXR agonists thereby enhancing biliary cholesterol excretion.[38] This, in combination with the reduction of apoB-lipoprotein synthesis, is believed to prevent the development of atherosclerosis,[39] suggesting that the activation of LXRs by cyanidin may promote biliary excretion of sterols via upregulating ABCG5.

On the other hand, SREBP1c is a LXR target gene that could induce hepatic fatty acid synthesis.[40] Potent LXR agonists such as T0901317 markedly stimulate lipogenesis in hepatocytes mainly via increased expression of SREBP1c which upregulates lipogenic enzyme expressions[41] leading to hepatic steatosis.[6,32] The current results show that cyanidin mildly activates LXRs and moderately upregulates the gene expression of SREBP1c (3.6-fold for 100 μM) in hepatocytes compared to those inductions by T0901317 (8.6-fold for 10 μM). Accordingly, effects on cellular lipid accumulation were compared further with cyanidin and T0901317.

Cyanidin reduces cellular lipid concentration in macrophages and lipid-loaded hepatocytes. LXRs are involved in hepatic lipid metabolism. Macrophage cells and lipid-loaded hepatocytes were stimulated with different cyanidin concentrations or T0901317 and cellular lipid levels were quantified[42] as reported previously.[43] Cyanidin significantly reduced concentrations of cellular cholesterol and TG in a dose-dependent manner in both THP-1-derived macrophages (Fig. 4A) and HepG2 cells (Fig. 4B). For example, cyanidin (100 μM) reduced cellular TG by −21% and −23% in THP-1 and HepG2 cells, respectively, whereas the full agonist of LXR T0901317 (10 μM) reduced cellular TG by −11% in THP-1 cells but increased cellular TG by 3.2-fold in HepG2 cells. These results

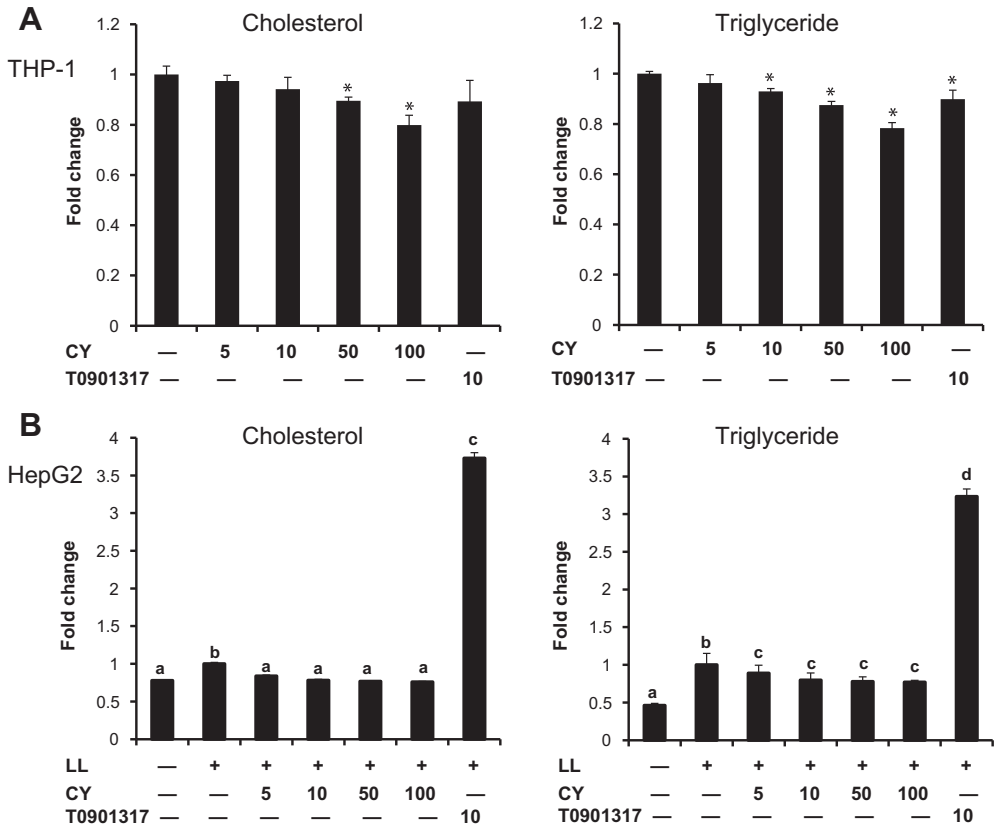

Figure 4. Cyanidin reduces intracellular cholesterol and TG concentrations in macrophage cells and lipid-loaded hepatocytes. (A) Cellular cholesterol and TG content in macrophage cells. *P <0.05 compared to controls; (B) cellular cholesterol and TG content in lipid-loaded (LL) hepatocytes. All data were analyzed by one-way ANOVA for repeated measures. Concentrations are shown in μM.

confirm that the activation of LXRs with cyanidin induced hypolipidemia not only in macrophage cells but in hepatocytes as well. Thus, LXR activation with cyanidin did not induce hepatic lipid accumulation, a common side effect of the full agonist T0901317.

In conclusion, it is suggested that cyanidin is a ligand of LXRs with a higher affinity for LXRα relative to LXRβ. The activation of LXRs by cyanidin reduced cellular TG and cholesterol concentrations not only in macrophage cells but also in lipid-loaded hepatocytes. Hence, cyanidin is a direct ligand for both LXRα and LXRβ and cellular lipid reduction via cyanidin stimulation operates at least in part through the modulation of cellular LXR activation and downstream gene expression.

Acknowledgments

This work was carried out with the support of Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ906950 and PJ00842203) Rural Development Administration, Republic of Korea. SPR instrument was provided by the Korea Basic Science Institute. We thank Professor Hyun-Gyu Song for the advice on FRET analysis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.05.030.

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19. CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium-F/12 (DMEM-F/12; Hyclone, Logan, UT, USA) at a density of 2×10^5/well in 24-well plates for 24 h. pCMV-LXR alpha or pCMV-LXRbeta (Benebiosis, Seoul, Korea)

was co-transfected with TK-LXRE-Luc. Transfection was performed with Hilymax (Dojindo, MD, USA) reagent according to the manufacturer's instructions for 24 h. Then the cells were stimulated with cyanidin (5–100 μM), T0901317 (2 μM) or vehicle (1% DMSO) for 24 h, and luciferase activity was quantified with a firefly luciferase assay kit (Biotium, Hayward, CA, USA).

20. The binding analyses of the immobilized hLXRα-LBD and hLXRβ-LBD to the ligands were measured using Biacore 2000 (GE Healthcare, U., Sweden). The LXR-LBD proteins were immobilized to the sensor chip CM5 (GE Healthcare, Uppsala, Sweden) with a coupling target of 8000–12,000 resonance units (RUs). Samples were automatically injected into flow cells at a concentration gradually decreasing from 100 to 0.78 μM. The increase in the SPR signal (expressed as RUs) induced by cyanidin or T0901317 was used to calculate the kinetics of the compounds using the BIAevaluation software version 3.1 (GE Healthcare, Uppsala, Sweden) via a 1:1 Langmuir binding fitting model.
21. The potential LXR-activating capacities of cyanidin, p.a. P., and phloroglucinaldehyde (PGA) were investigated using LanthaScreen™ TR-FRET LXRα and LXRβ coactivator assays (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Increasing concentrations of samples were added to the LXR-LBD and the co-activator peptides: fluorescein-TRAP220/DRIP-2 for LXRα and fluorescein-D22 for LXRβ with DMSO (1%) was used as a vehicle. After 2 h incubation at room temperature, the 520/495 TR-FRET ratio was measured using a Spectra Max instrument with TRF laser excitation. Binding curves were generated by plotting the emission ratio vs. the log [ligand]. To determine EC_{50} values, data were fit using the equation for a sigmoidal dose–response (varying slope), as provided in GraphPad™ Prism® 5.0.
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