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Cyanidin is an agonistic ligand for peroxisome proliferator-activated receptor-alpha reducing hepatic lipid

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ABSTRACT

To investigate the underlying mechanism of targets of cyanidin, a flavonoid, which exhibits potent anti-atherogenic activities *in vitro* and *in vivo*, a natural chemical library that identified potent agonistic activity between cyanidin and peroxisome proliferator-activated receptors (PPAR) was performed. Cyanidin induced transactivation activity in all three PPAR subtypes in a reporter gene assay and time-resolved fluorescence energy transfer analyses. Cyanidin also bound directly to all three subtypes, as assessed by surface plasmon resonance experiments, and showed the greatest affinity to PPARα. These effects were confirmed by measuring the expression of unique genes of each PPAR subtype. Cyanidin significantly reduced cellular lipid concentrations in lipid-loaded steatotic hepatocytes. In addition, transcriptome profiling in lipid-loaded primary hepatocytes revealed that the net effects of stimulation with cyanidin on lipid metabolic pathways were similar to those elicited by hypolipidemic drugs. Cyanidin likely acts as a physiological PPARα agonist and potentially for PPARβ/δ and γ, and reduces hepatic lipid concentrations by rewiring the expression of genes involved in lipid metabolic pathways.

1. Introduction

The peroxisome proliferator-activated receptor (PPAR) is a nuclear hormone receptor, which is made up of transcriptional factors activated by the binding of ligands to their ligand-binding domains (LBDs) [1]. Although the three PPAR subtypes, alpha (α), delta/beta (δ/β), and gamma (γ), display different cellular distributions and distinct pharmacological profiles, they are all essentially related to lipid and glucose metabolism [2]. Because PPARs share similar structures within the LBDs, synthetic ligands that simultaneously activate at least two of the PPAR subtypes represent potent candidate drugs for the treatment of abnormal metabolic homeostasis by improving clinical symptoms of cardiovascular disease, type 2 diabetes mellitus (T2DM), and obesity [3,4].

PPAR activation provides a number of metabolic benefits. However, potent full agonists can induce significant side effects. For example, rosiglitazone, induces carcinogenesis, edema, and cardiovascular complications, and thus its use in newly diagnosed T2DM has been suspended since 2010 [5]. In addition, the recent focus in this area has centered on the concept of selective PPAR modulators, particularly for PPARγ, following the observation that the full agonists such as rosiglitazone and pioglitazone are counterbalanced by receptor-mediated side effects [6]. A partial agonist could retain efficacy but reduce the transcriptional regulation thought to be responsible for the attendant side effects.

Considering the beneficial pharmacological effects of the PPAR subtypes, the concept of simultaneously but moderately activating all PPAR subtypes with a single compound, for example, pan- or dual-agonists, could be advantageous for the prevention and treatment of the clinical symptoms of ectopic fat accumulation, including liver steatosis, diabetes mellitus, and metabolic syndrome. Simultaneous activation of all PPAR subtypes may also reduce the occurrence of adverse side effects, which are often associated with full PPAR agonists [7].

Initially, we screened approximately 900 Korean natural extracts and compounds from plant and marine organisms for PPAR agonist or antagonist activity using a reporter gene assay, and found that cyanidin exhibited potent PPAR activity. Cyanidin is a well-defined anthocyanidin abundant in fruits, and vegetables [8] with various biological activities, including potent antioxidant, radical-scavenging, hypolipidemic, and anti-inflammatory activities. Therefore, cyanidin protects against cellular oxidative damage and reduces plasma and

Abbreviations: PPAR, peroxisome proliferated-activated receptor; LBD, ligand-binding domain; RXR, retinoid X receptor; Apo, apolipoprotein; K_D, equilibrium dissociation constant; EC_{50}, the half maximal effective concentration; SPR, surface plasmon resonance; TR-FRET, time-resolved fluorescent resonance energy transfer; PCA, procatechic acid; PGA, phloroglucinaldehyde

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cellular accumulation of undesirable lipids, collectively reducing the risk of age-related metabolic diseases, such as cardiovascular diseases, diabetes mellitus, and obesity [9].

Some evidence also suggests that foods with cyanidin and its glycosides inhibit the formation of atherogenic lipoprotein and oxidized low-density lipoprotein (LDL) particles *in vitro* [10,11] and *in vivo* [12–15]. Several studies have shown that cyanidin-rich diet results in significantly reduced cholesterol, triglyceride (TG), and ApoB plasma concentrations and a decreased aortic fatty streak area [16], indicating that it may also modulate lipid metabolism. In addition, several studies have indicated that a cyanidin-rich diet suppresses the high fat diet-induced increase in body weight gain, hyperglycemia, and hyperinsulinemia by inducing adipokine secretion [17] and the upregulation of hormone-sensitive lipase [18]. However, although much data suggest a hypolipidemic effect of cyanidin both *in vitro* and *in vivo*, the direct molecular target remains unknown. We investigated the direct interaction between cyanidin and LBD of PPAR proteins, and examined its hypolipidemic effects in terms of regulation of PPAR target gene expression using selected biomarkers and transcriptome analyses.

2. Materials and methods

2.1. Reagents

Cell culture reagents and supplies were obtained from Hyclone (Logan, UT, USA). Cyanidin (molecular weight is 287.24 g/mol Fig. 1A) was purchased from Extrasynthese (Genay, Cedex, France), fenofibrate, troglitazone, and GW9662 were purchased from Sigma (St. Louis, MO, USA). Lovastatin was purchased from Chungwai Pharmaceutical Company (Gyunggi, Korea). GW0742 was purchased from the Cayman Chemical Company (Ann Arbor, Michigan, USA). Total RNA extraction reagent (RNAiso Plus) and real-time polymerase chain reaction (PCR) premix (SYBR® Premix Ex Taq™) were obtained from Takara (Otsu, Japan). The Oligo $(dT)_{15}$ primer was purchased from Promega (Madison, WI, USA).

2.2. Cell culture and lipid staining

HepG2 and CHO-K1 cells were obtained from the Korean Cell Line Bank (Seoul, Korea), and were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) and Dulbecco's modified Eagle's medium-F/12 (DMEM-F/12; Hyclone, Logan, UT, USA) medium, respectively, containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (PEST; Welgene Inc., Seoul, Korea). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2.

HepG2 cells were cultured in six-well culture plates for 24 h. Medium was removed and the cells were washed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) twice. Then the cells were treated with free fatty acids (palmitic acid and oleic acid, 400 μM each) with 0.5% bovine serum albumin (BSA, Bovogen Biologicals, Melbourne, Australia) for 24 h, followed by cyanidin (5, 10, 50, or 100 μM) or 10 μM fenofibrate, 1 μM GW0742, and 10 μM troglitazone for a further 24 h, with 1% DMSO as a vehicle. The treated HepG2 cells were stained with oil red O or 5 μL/mL of normal growth medium of DiI dye (Invitrogen, Carlsbad, CA, USA), following the general protocol described previously [19,20]. Cells stained by oil red O was imaged under a Nikon ECLIPSE Ti-S microscope (Nikon, Japan), and cells stained with DiI was imaged under a LSM 5 Exciter Confocal laser scanning microscope (Carl-zeiss, Petaluma, CA, US) at absorption of 549 nM and fluorescence emission maxima of 565 nM.

HepG2 cells were washed twice with 1 mL PBS, treated with 1 mL hexane/isopropanol (2:1) for 30 min at room temperature, and then

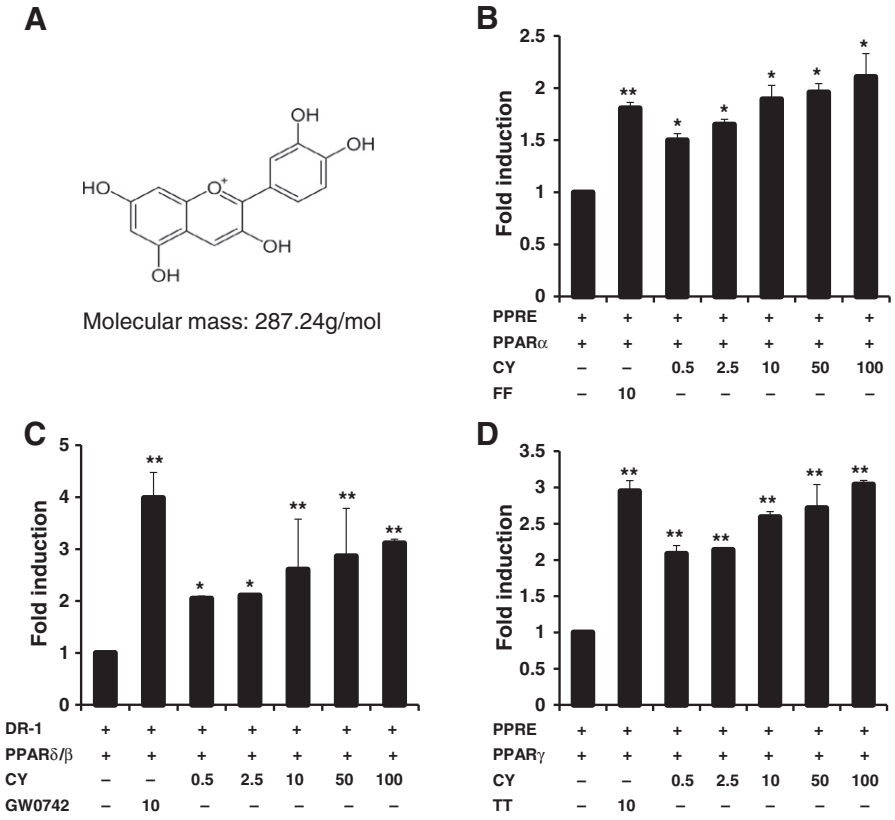


Fig. 1. Cyanidin induces transactivation of PPARα, PPARδ/β, and PPARγ. A. Structure of cyanidin. B–D. Effects of cyanidin on the transactivation of PPARα (B), PPARδ/β (C), and PPARγ (D). Data represent the relative fold increase compared to the non-treated control; all determinations were performed in triplicate. *, $P<0.05$ and **, $P<0.001$ compared to controls.

transferred to test tubes. The wells were again washed with 1 mL hexane/isopropanol, and the washing solutions were transferred to the corresponding test tubes. The organic solvent was removed using a vacuum centrifuge, and the lipids were resuspended in 95% ethanol. Intracellular total cholesterol and triglyceride concentrations were quantified *via* an enzymatic method using a Cobas C111 automatic analyzer (Roche Diagnostic Systems Inc., Indianapolis, IN, USA), as reported previously [21]. Lipid levels were normalized to the total cellular protein concentration as determined using the BCA protein assay (Pierce Biotechnology, Rockford, USA).

2.3. Transfection and luciferase assay

CHO-K1 cells were cultured in DMEM-F/12 (Hyclone, Logan, UT, USA) in 24-well plates at a density of 2×10^5/well. pSG5-PPAR alpha or pBABE-zeo-PPAR gamma2 (Addgene, MA, USA) was co-transfected with pCMV-3xPPRE-Luc. pAdTrack-CMV-PPARδ/β (Addgene, MA, USA) was co-transfected with pCMV-DR-1. Transfection was performed with Hilymax (Dojindo, MD, USA), according to the manufacturer's instructions. At 24 h post-transfection, cells were stimulated with cyanidin (0.5 to 100 μM) or vehicle (1% DMSO) for 24 h, and luciferase activity was quantified with a firefly luciferase assay kit (Biotium, Hayward, CA, USA).

2.4. Modeling method

The binding properties of cyanidin were analyzed as described previously [22]. Briefly, the crystal structure of the PPARα:RXR-β complex bound to T0901317 (PDB ID: 1UHL) was used as the target for docking calculations. The structure of cyanidin was initially generated using the program Maestro v7.0 (Portland, OR, USA). Cyanidin was energy-minimized with the molecular mechanics (MM3*) force field prior to docking in MacroModel v8.1 [23]. Docking calculations were carried out with the Glide software (Schodinger, Portland, OR) [24]. The best-docked pose was selected and scored from the calculated binding affinity: the receptor–ligand molecular mechanics interaction and ligand strain energies. Furthermore, the poses were subjected to another final optimization procedure in which the receptor–ligand complex underwent a full molecular mechanics energy minimization (OPLS–AA force field) to optimize flexible PPAR side-chain residues that could interact with ligands. The lowest-energy poses obtained in this fashion were subjected to a Monte Carlo procedure to obtain the final docking solution.

2.5. Cloning, expression, and purification of PPAR proteins

The human PPARα LBD (amino acid residues 280–468), PPARδ/β LBD (residues 254–441), and PPARγ LBD (residues 317–505) were cloned into the expression vector pET-32a-c(+) (Novagen, Darmstadt, Germany) and thereby fused to an N-terminal hexa-histidine tag with a thrombin cleavage site. Transformed E. coli Rosetta (DE3) was cultured overnight and then inoculated into LB broth (1 L) containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L), and cultured at 37 °C for 4 h. Then the expression of the PPAR LBD proteins was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 mM, Bio Basic Inc. Ontario, Canada), the temperature was lowered to 18 °C, and the cells were cultured for an additional 12 h. Cells were harvested by centrifugation (4000×g, 15 min, 4 °C), and the pellets were resuspended in lysis buffer (50 mM Tris, 5 mM β-mercaptoethanol, pH 7.1) and disrupted by sonication on ice for 3 min at 1 s sonication in 5 s intervals. The lysate was centrifuged (17,000× g, 20 min, 4 °C) and the supernatant was collected and passed through a 0.45 μm filter (Millpore Corrigtwohill, Co. Cork, Ireland.). The filtrate was loaded onto a HiTrap™ Chelating HP Column (GE Healthcare, Giles, Bucks HP8 4SP, UK) at a flow rate of 5 mL/min, then washed with 10 column volumes of lysis buffer, and subsequently with five column volumes of a mixture of 92% lysis buffer and 8% elution buffer (50 mM Tris, 5 mM beta-mercaptoethanol with 500 mM imidazole, pH 7.1). The lysis buffer initially contained 8% elution buffer; this was gradually increased to 70% at 30 min by a gradient elution program using a Bio-Rad BioLogic LP system (Hercules, CA, USA). Buffer exchange for the purified proteins was performed using a concentrator (Sartorius stedim, Aubagne Cedex, France) against PBS buffer, and stored at −80 °C for further use.

2.6. Surface plasmon resonance (SPR)

Binding analyses of immobilized hPPARα LBD, hPPAR δ/β LBD, and hPPARγ LBD to ligands were conducted using a Biacore 2000 instrument (GE Healthcare, Uppsala, Sweden). Immobilization of the proteins to the hydrophilic carboxymethylated dextran matrix of the sensor chip CM5 (GE Healthcare, Uppsala, Sweden) with a coupling target of 8000–12000 resonance units (RUs) was performed using the standard primary amine coupling reaction. The proteins to be covalently bound to the matrix were diluted in 10 mM sodium acetate buffer (pH 4.0) to a final concentration of 45–200 mg/mL. Equilibration of the baseline was achieved using a continuous flow of HBS running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% [v/v] surfactant P20, pH 7.4) containing 1% DMSO through the chip for 1–2 h. Samples were automatically injected into flow cells at a concentration gradually decreasing from 100 to 0.78 μM. All Biacore data were collected at 25 °C using HBS with 1% DMSO as the running buffer at a constant flow of 25 μL/min. After injection into the SPR system, the sample binds the PPAR protein, resulting in an increase in the SPR signal (expressed as RUs), which is presented graphically as a function of time in the sensorgrams. After the desired association time, a solution lacking sample (usually buffer) is injected into the microfluidics that dissociates the bound complex consisting of the sample and each PPAR LBD subtype. As the sample dissociates from the bait ligand, a decrease in the SPR signal (RU) is observed. From these association ('on rate,' k_a) and dissociation ('off rate,' k_d) rates, the equilibrium dissociation constant ('binding constant,' K_D) was calculated using the BIAevaluation software version 3.1 (GE Healthcare, Uppsala, Sweden), and a 1:1 Langmuir binding fitting model was used to determine the equilibrium dissociation constants of cyanidin, fenofibrate, GW0742, and troglitazone binding.

2.7. Cell-free time-resolved fluorescence resonance energy transfer (TR-FRET) assays

LanthaScreenTM TR-FRET Co-activator Assays (Invitrogen, Carlsbad, CA, USA) were used to identify cyanidin agonist activity to PPARα, PPARδ/β, and PPARγ. Test compounds were diluted in DMSO, and the assays were run according to the manufacturer's instructions. To test the ability of a molecule to function as an agonist, increasing concentrations of cyanidin or control agonists were added to PPAR LBD and the following co-activator peptides were added to the solution: fluorescein-PGC1α for PPARα, fluorescein-C33 for PPARδ/β, and fluorescein-TRAP220/DRIP-2 for PPARγ. 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 and the following filter sets: excitation 340 nm, emission 495 nm, and emission 520 nm. A 100 μs delay followed by a 400 μs integration time was used to collect the time-resolved signal. Results were calculated by dividing the emission signal at 520 nm by that at 495 nm. 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.

2.8. Quantitative real-time PCR

HepG2 cells were cultured in DMEM containing 10% FBS and 1% PEST in six-well culture plates at a density of 10^6/well for 24 h, and

then treated with 5, 10, 50, or 100 μM cyanidin or fenofibrate (10 μM), GW0742 (1 μM), and troglitazone (10 μM) for a further 24 h. DMSO (1%) was used as a vehicle. Then 1 mL total RNA extraction reagent RNAiso plus (Takara, Shiga, Japan) was added, and total RNA was extracted following the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg total RNA using M-MLV reverse transcriptase, oligo-dT, and dNTPs (Mbiotech, Korea). Then the cDNAs were used as templates for PCR reactions. Specific primers were designed using the OligoPerfect™ Designer program (Invitrogen, Carlsbad, CA, USA). PCR reactions were run on a Bio-Rad iQ5 iCycler system (Bio-Rad, Hercules, CA, USA) using RealMasterMix SYBR ROX reagent (5 PRIME, Hamburg, Germany). The PCR reaction conditions were: 95 °C for 3 min followed by 50 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 60 s. A melting curve of 71 cycles, starting at 55 °C and increasing by 0.5 °C every 10 s was performed to determine primer specificity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of data, and gene expression levels were calculated according to the iQ5 Optical System Software (version 2; Bio-Rad, Hercules, CA, USA).

2.9. Oligonucleotide microarray analysis

HepG2 cells were cultured in DMEM medium containing 10% FBS and 1% PEST in six-well culture plates at a density of 5×10^5/well for 24 h, and then were treated with DMEM containing 10% FBS, 1% PEST, and 400 μM of oleic acid and BSA (oleic acid to BSA ratio, 6.6:1) to induce lipid droplets for 24 h. Then the lipid was removed, and to avoid interference with intracellular gene expression by serum lipoproteins, cells were incubated in serum-free DMEM medium supplemented with or without 100 μM cyanidin, 10 μM fenofibrate (FF), or 10 μM lovastatin (ST) for an additional 24 h. The vehicle was DMSO (1%). We also treated HepG2 cells with 100 μM cyanidin, 10 μM FF, or 10 μM ST directly for 24 h without lipid loading, with 1% DMSO treatment as the vehicle.

Two-color microarray experiments were performed using HepG2 cells. Total RNA was isolated using the total RNA extraction reagent RNAiso Plus, purified with the RNase-Free Dnase I set (Qiagen, Valencia, CA, USA), and further cleaned up with the Ribo-clear kit (GeneAll, Korea). For cDNA synthesis, reverse transcription was performed with 8 μg RNA, M-MLV reverse transcriptase, oligo-dT, and dNTPs (Mbiotech, Korea). cDNA was labeled with the monofunctional dyes Cy3-dUTP or Cy5-dUTP (GeneChem Inc., Korea) during reverse transcription. After the reactions, dye-labeled samples were purified using the QIAquick PCR purification Kit (Quiagen, Valencia, CA, USA) and dried in a speedvac (N-Biotek Inc., Korea). Resuspended cDNAs in hybridization buffer were hybridized onto Human OneArrays (Phanlanx Biotech Group, Hsinchu, Taiwan), containing 29,187 human genome probes. The arrays were scanned with a GenePix 4000B (Axon Instruments, Palo Alto, CA, USA), and the data were visualized using the GenePix Pro 5.1 software (Axon Instruments, Palo Alto, CA, USA). The signals of each probe spot were computed with the GenePix Pro 5.1 software, and the background intensities were subtracted from those of the probes. Then the probes were normalized using the Acuity 4.0 software (Axon Instruments, Palo Alto, CA, USA). Transcriptional responses to cyanidin stimulation were analyzed using the two-class unpaired method of the significance analysis of microarrays (SAM), as implemented in the Bioconductor samr package. To investigate the effect of cyanidin on various metabolic pathways, genes were selected based on gene ontology [25] and KEGG [26,27], and categorized into gene sets. The following gene sets were used: cholesterol biosynthesis, bile acid synthesis, fatty acid biosynthesis, fatty acid metabolism, and the TCA cycle. Expression values of genes in the gene sets were visualized by heatmapping using GenePattern [28], and displayed as red (highest) to blue (lowest). White represented a fold change of one. Gene sets were arranged in descending order to compare the white portion to that of the vehicle.

2.10. Statistical analysis

Data are presented as mean + SEM. Student's *t*-test was used to compare the means of two groups. Statistical differences between experimental groups and the DMSO control and lipid-loaded group were calculated using a one-way ANOVA. A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Cyanidin is a ligand for all three PPAR subtypes

In the reporter gene assay, cyanidin potently activated PPAR transactivation in a dose-dependent manner. PPARα transactivation was induced 1.9-, 2.0-, and 2.1-fold at 10, 50, and 100 μM, respectively, compared to the PPARα agonist fenofibrate (Fig. 1B). Cyanidin also induced the activation of PPARδ/β in a dose-dependent manner by approximately 2.6-, 2.9-, and 3.1-fold at 10, 50, and 100 μM, respectively (Fig. 1C). Cyanidin induced PPARγ transactivation 2.6-, 2.7-, and 3.0-fold compared to controls at 10, 50, and 100 μM, respectively (Fig. 1D). These findings suggest that cyanidin activates the transactivation of all three PPAR subtypes.

Cyanidin, fenofibrate, GW0742, and troglitazone were analyzed with SPR-Biacore for direct interactions with PPAR LBD proteins. Interestingly, the data indicated that cyanidin directly associated with the three PPAR subtypes (Fig. 2). The K_D of cyanidin to PPARα was 3.08 μM, that to PPARδ/β was 389.01 μM, and that to PPARγ was 24.50 μM (Table 1). Thus, cyanidin directly bound to all three PPAR subtypes, with the highest affinity for PPARα, followed by PPARγ and PPARδ/β.

To further investigate whether cyanidin functions as an agonistic ligand for the three PPAR subtypes, TR-FRET analyses were performed. The co-activators of PPARs were PGC1α for PPARα, Fluorescein-C33 for PPARδ/β, and Fluorescein-TRAP220/DRIP-2 for PPARγ. Cyanidin activated all three PPAR subtypes (Fig. 3A-C). The half maximal effective concentration (EC_{50}) values, which represent the ability of cyanidin to activate PPARα, PPARδ/β, and PPARγ, were 3.03, 597.53, and 28.03 μM, respectively (Table 2). After binding with the three PPAR subtype LBDs, cyanidin showed an agonistic effect against all three subtypes.

To gain insight into the determinant of cyanidin binding affinity, cyanidin was docked into the PPARα receptor LDB. In virtual modeling, cyanidin bound to PPARα though hydrogen bonding *via* its hydroxyl and amino groups (Fig. 3D). The hypothetical modeling suggests potential interaction of 4'-hydroxyl group of cyanidin with either Ser-280 in helix 3, Tyr-464 in helix 12 (AF-2), or Tyr-314 in helix 4.

3.2. Cyanidin degradation products do not bind PPARs

It has been suggested that a significant portion of dietary cyanidin is degraded into procatechic acid (PCA) and phloroglucinaldehyde (PGA) [29]. Thus, we further analyzed whether these degradation products could directly associate with PPAR LBDs. In both SPR analyses and TR-FRET assays, neither PCA nor PGA displayed significant binding activity to all three PPAR LBDs (Fig. 4A–B), and none of them recruited a co-activator peptide to the PPAR LBD (Fig. 4C–D). These data suggest that cyanidin degradation products do not directly affect PPAR activity.

3.3. Cyanidin reduces cellular lipid concentration in lipid-loaded hepatocytes

Each of the three PPAR subtypes is involved in hepatic lipid metabolism. The results showed that cyanidin (100 μM) significantly reduced concentrations of cellular cholesterol (−24%) and TG (−23%) in lipid-loaded HepG2 cells in a dose-dependent manner (Fig. 5A–B). These results confirm that activation of PPARs with cyanidin induced a hypolipidemic effect in hepatocytes. Both the oil red O- and DiI-lipid

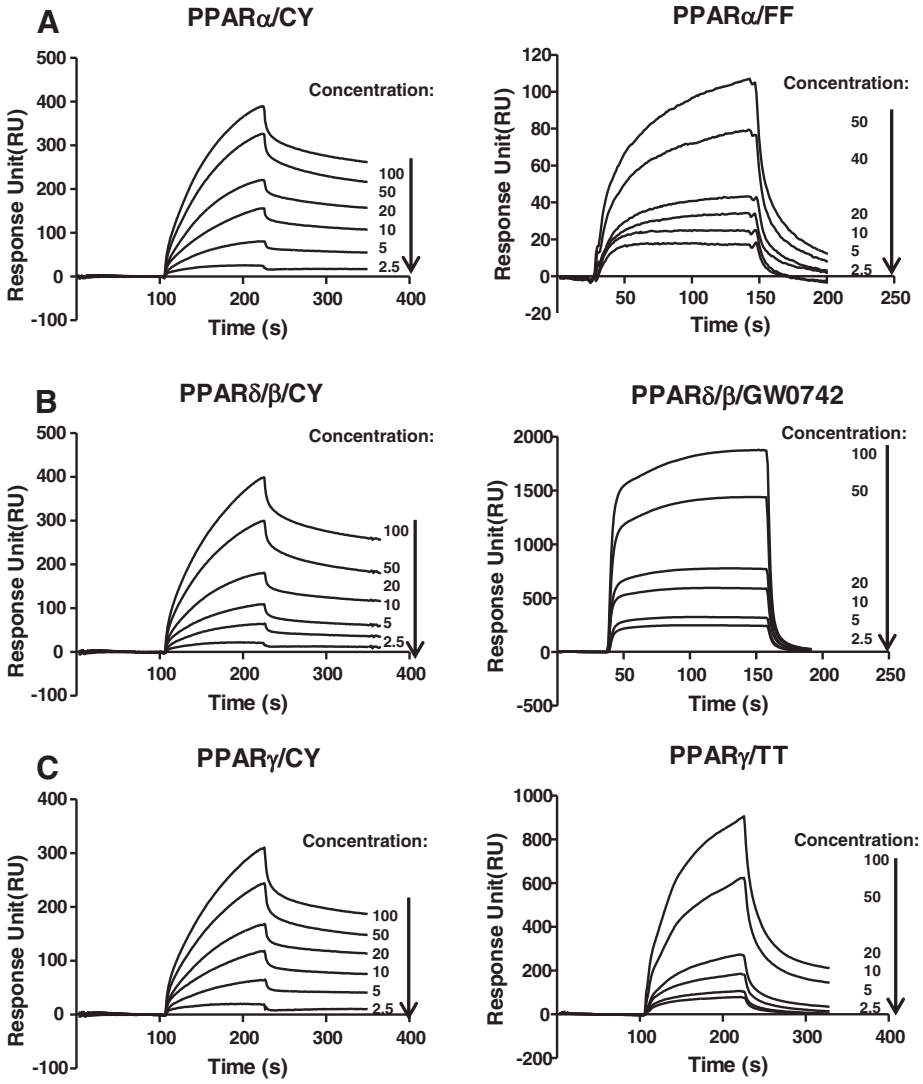


Fig. 2. Cyanidin binds directly to PPARα, PPARδ/β, and PPARγ LBD. SPR sensorgrams were obtained from Biacore after injection of a series of concentrations of cyanidin (CY, left panel of A, B and C), fenofibrate (FF, right panel of A), GW0742 (right panel of B), or troglitazone (TT, right panel of C) over the immobilized hPPARα LBD, hPPARδ/β LBD, or hPPARγ LBD response.

staining results also indicated that stimulation of hyperlipidemic HepG2 cells with cyanidin resulted in reduced lipid accumulation (Fig. 5C–D). The effects of cyanidin were comparable to those of the PPAR agonists, fenofibrate, GW0742, and troglitazone.

3.4. Cyanidin regulates the transcription of PPARα-, PPARδ/β-, and PPARγ-specific responsive genes

In hepatocytes stimulated with cyanidin, PPARα transcription was increased by 3.8-fold (50 μM) and 6.3-fold (100 μM). Moreover, expression of Apo-AI was upregulated and that of Apo-CIII and HMGCS2, which are unique target genes for PPARα, was downregulated (Fig. 6A). PPARδ/β expression was induced by cyanidin by 3.2-fold (50 μM) and 5.0-fold (100 μM). Furthermore, expression of its unique target genes, including ILK, PDK1, and UBC, was also upregulated (Fig. 6B). Cyanidin also induced PPARγ gene expression by 2.1-, and 2.2-fold at 50 μM, and 100 μM, respectively, and that of the PPARγ responsive genes, GyK and PEPCK (Fig. 6C).

Table 1
Equilibrium dissociation constants (K_D) of cyanidin and synthetic PPAR agonists to hPPARα LBD, hPPARδ/β LBD, and hPPARγ LBD, quantified using SPR-Biacore.

Compound	Equilibrium dissociation constant (K_D[a])		
	hPPARα LBD	hPPARδ/β LBD	hPPARγ LBD
Cyanidin	3.08 μM	389.01 μM	24.50 μM
Fenofibrate	301.02 nM	–	–
GW0742	–	44.80 nM	–
Troglitazone	–	–	254.03 nM

[a] K_D, equilibrium dissociation constants. K_D values were calculated from the association ('on rate,' k_a) and dissociation ('off rate,' k_d) rates by using the BIAevaluation software version 3.1 (Biacore).

3.5. Global effects of cyanidin on the transcriptome of lipid-loaded HepG2 cells

The genome-wide hypolipidemic effects of cyanidin in lipid-loaded hepatocytes were investigated by comparison to those of two lipid-lowering drugs, lovastatin and fenofibrate. Pathway analysis revealed that cyanidin (100 μM) inhibited the cholesterol biosynthesis pathway and stimulated the bile acid synthesis pathway to a lesser degree than did lovastatin (10 μM). The expression of genes involved in fatty acid biosynthesis, fatty acid metabolism, and the tricarboxylic acid cycle were regulated by cyanidin in a similar manner to fenofibrate (10 μM), suggesting that high cyanidin levels regulate the transcription of hepatic fatty acid metabolism genes in a manner similar to fenofibrate. Furthermore, the expression of target genes of all PPAR subtypes including

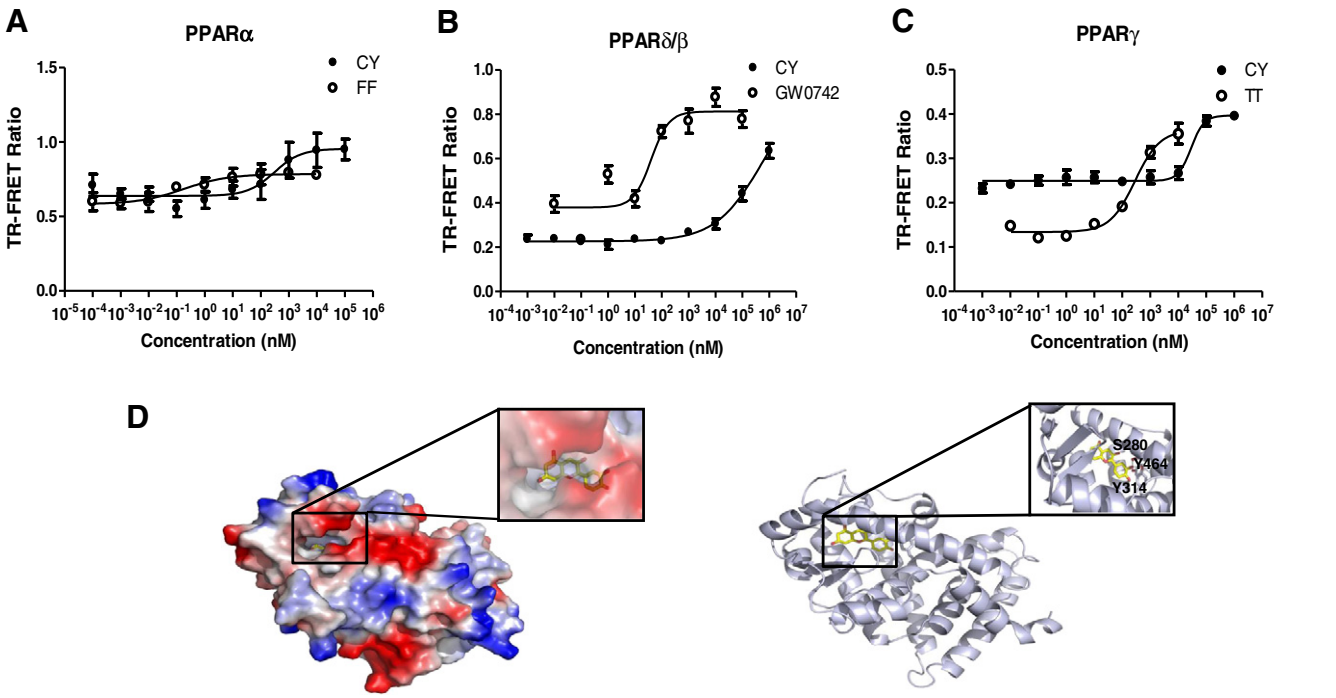


Fig. 3. Cyanidin binding induces recruitment of co-activator peptides by activation of PPARα, PPARδ/β, and PPARγ in a TR-FRET assay. A. Cyanidin (CY) and fenofibrate (FF) were incubated with PPARα LBD. B. Cyanidin and GW0742 were incubated with PPARδ/β LBD. C. Cyanidin and troglitazone (TT) were incubated with PPARγ LBD. Increasing concentrations (nM) of cyanidin or control PPAR agonists were incubated with PPAR LBD and the corresponding co-activator peptide described in the Materials and methods. D.** The proposed complex of cyanidin with the ligand-binding pocket of PPARα. The ribbon structure displays the hydrogen bonds between cyanidin and S280 (H3), Y434 (H4), and Y314 (H12) in AF-2 of PPARα.

SCP2, ACADVL, and ACADM, were induced. The common target gene CYP27A1, which is oppositely regulated by PPARα and PPARγ, was significantly induced, suggesting a dominant role of PPARα over the γ-form in CYP27A1 transcription (Fig. 7A). Microarray results suggested that cyanidin modulated all three PPAR subtypes. A list of the full names of the genes included in these pathways is shown in Supplemental Table 1. To confirm the microarray results, we selected 12 genes involved in lipid and glucose metabolism and quantified their expression by qPCR. The qPCR and microarray data regarding expression of these genes were comparable (Supplemental Fig. 1).

Stimulation of lipid-loaded HepG2 cells with cyanidin rewires the hepatic transcriptome profile, with the effects of cyanidin being comparable to those of lovastatin and fenofibrate. We selected 1231 genes expressed in all experimental groups, including control and lipid-loaded hepatocytes and lipid-loaded cells stimulated with cyanidin, fenofibrate, or lovastatin, and compared their transcriptome profile after treatment to cyanidin and other hypolipidemic drugs. Lipid-loading of hepatocytes dramatically altered their gene expression patterns; 65.0% of the selected genes showed a >20% change in expression (fold change >1.2 [red] or <0.8 [blue]). However, stimulation of lipid-loaded cells with cyanidin resulted in the expression of 31.2% of the selected genes being altered by >20%. Thus, 33.8% of the selected genes exhibited <20% change in expression after stimulation with cyanidin compared to the lipid-loaded condition. Stimulation with fenofibrate and lovastatin altered the expression of 31.0% and 27.3% of the selected genes by >20%, respectively. Thus 34.0% and 37.7% of the selected genes fell into the <20% change category compared to the lipid-loaded condition after stimulation with fenofibrate and lovastatin, respectively (Fig. 7B). Lovastatin exhibited the most potent normalizing effect on gene expression (<20% compared to the control) in lipid-loaded hepatocytes. These results indicate that the effects of dietary cyanidin on the hepatic transcriptome may be comparable to those of lovastatin and fenofibrate.

Table 2
Half-maximal effective concentrations (EC_{50}) of cyanidin and synthetic PPAR agonists to hPPARα LBD, hPPARδ/β LBD, and hPPARγ LBD, assessed by cell-free FRET assay.

Compound	Half maximal effective concentration (EC_{50}[a])		
	hPPARα LBD	hPPARδ/β LBD	hPPARγ LBD
Cyanidin	3.03 μM	597.53 μM	28.03 μM
Fenofibrate	446.30 nM	–	–
GW0742	–	40.37 nM	–
Troglitazone	–	–	283.50 nM

[a] Binding curves were generated by plotting the emission ratio *vs.* the log [ligand], and EC_{50} values were calculated using the equation for a sigmoidal dose response (varying slope), as provided in GraphPad™ Prism® 5.0.

4. Discussion and conclusion

4.1. Discussion

Ligand-induced activation of PPARs controls the expression of innumerable genes involved in lipid and lipoprotein metabolism, the activation of which could prevent or ameliorate clinical symptoms of hyperlipidemia, insulin resistance, obesity, and hepatic steatosis. Furthermore, because of the considerable side effects caused by individual PPAR agonist, recent efforts have focused on identifying a compound derived from natural sources with moderate binding affinity for two or more PPAR isoforms for the development of balanced ligands [30,31].

Here we demonstrate that cyanidin, binds directly to all three subtypes of PPAR isoforms with the strongest affinity to PPARα, and that binding induces common agonistic effects, which, to the best of our knowledge, is unique. Cyanidin is a moderate agonist for PPARα, and could be a weak agonist for PPARγ and PPARβ/δ. Thus, the biological effects of cyanidin on lipid metabolism, insulin sensitivity, inflammation, and obesity may be mediated by PPAR activation in metabolically active tissues.

Structural studies have suggested that the three PPAR subtypes share a similar general mechanism of activation, with the bound ligand stabilizing the C-terminal activating helix 12 (AF-2) by making

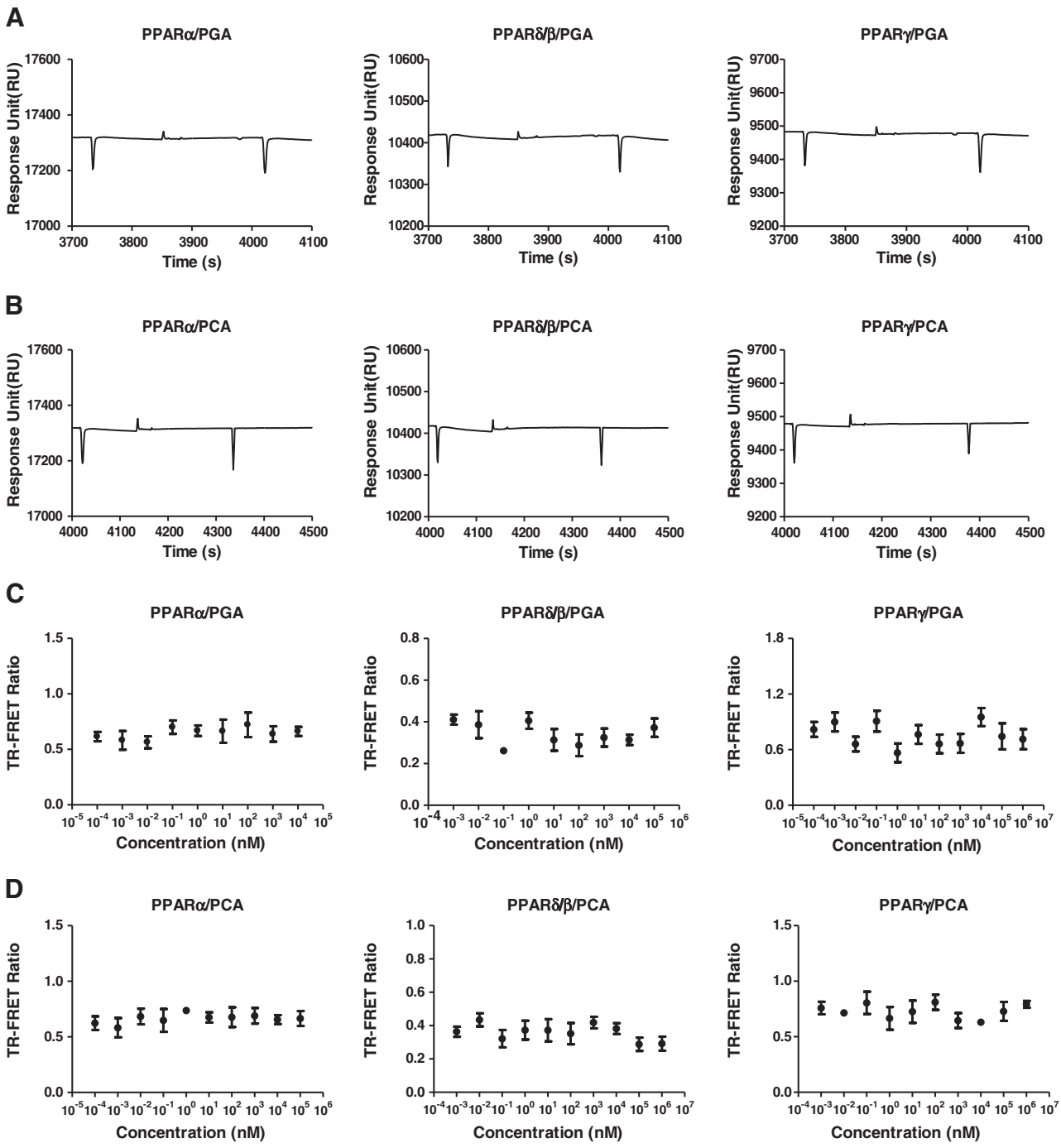


Fig. 4. Phloroglucinaldehyde (PGA) and protocatechuic acid (PCA) are neither ligands nor agonists of PPARα, PPARδ/β, and PPARγ, as assessed using SPR and cell-free FRET assays. A. Sensorgrams were obtained by injection of PGA (100 μM) over the immobilized hPPARα LBD, hPPARδ/β LBD, or hPPARγ LBD response. B. Sensorgrams obtained by injection of PCA (100 μM) over the immobilized hPPARα LBD, hPPARδ/β LBD, or hPPARγ LBD response. C. TR-FRET ratio of PGA for PPAR co-activator recruitment. D. TR-FRET ratio of PCA for PPAR co-activator recruitment.

hydrophobic contacts with it, thereby allowing the binding of co-activators [32]. The ligand-binding sites of PPAR LBDs exhibit Y-shaped pockets. The deepest arm is located behind helix 3, where the bound ligand contacts both hydrophilic and hydrophobic residues [32,33]. In particular, the hydrophobic patch contains the Tyr residues (Y314 and Y464) within the AF-2 helix, which plays a key role in ligand interactions [32,34]. Our hypothetical model of PPARα suggests that the 4′-hydroxyl group of the cyanidin ring potentially interacts with the key residues (Y314 and Y464) in the AF-2 helix, Y464, suggesting agonistic modulation of PPAR activation by cyaniding (Fig. 3D).

The hydrophobic arm in the ligand-binding pocket has been suggested to play a crucial role in the different ligand-binding affinities to the three PPAR LBDs. Three-dimensional structural studies have revealed that compared to the bulkier Cys275 and Arg284 side chains in the hydrophobic arm of PPARα, PPARγ has a smaller Gly284 side chain in the ligand-binding pocket [32]. The ligand-binding cavity of PPARδ/β is significantly narrower in the region adjacent to the AF-2 helix, such that PPARδ/β may have a stricter ligand specificity than PPARα, barely accommodating bulky ligand substituents in the region that comes into contact with the LBD cavity [32]. Collectively, the subtle structural differences among PPARs may affect

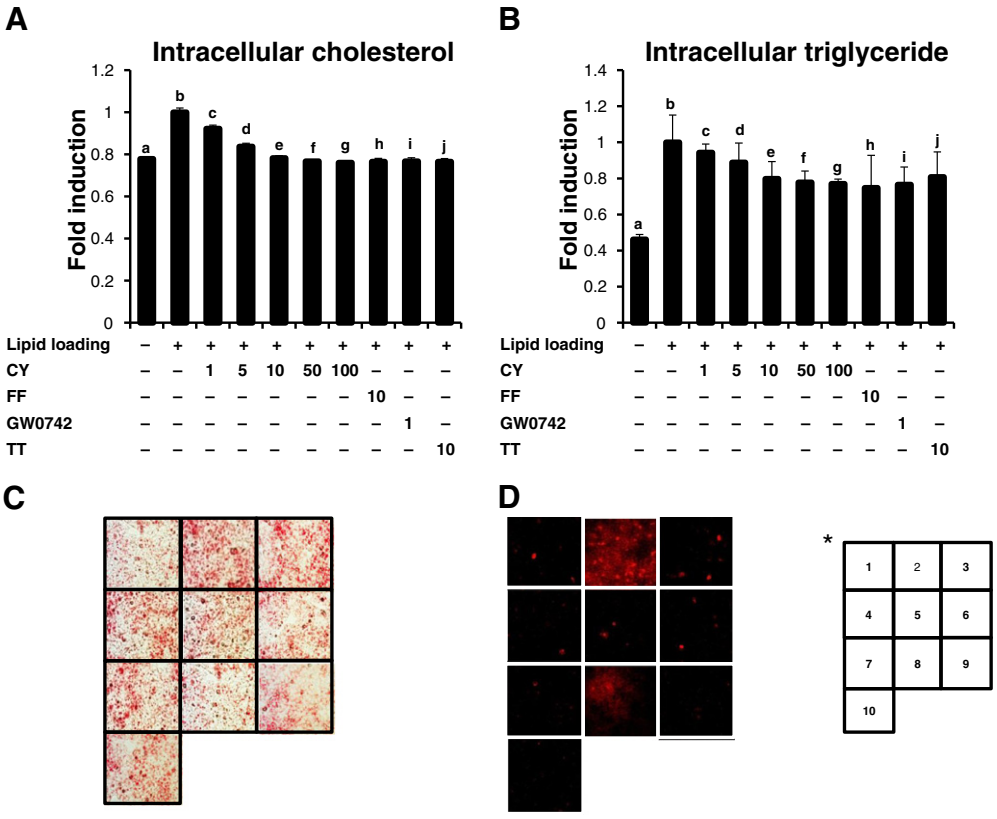


Fig. 5. Cyanidin (CY) reduces intracellular cholesterol and triglyceride concentrations in lipid-loaded hepatocytes. A. Cellular cholesterol content. B. Cellular triglyceride content. Lipid concentrations are relative to those of lipid-loaded cells. All data were analyzed by one-way ANOVA for repeated measures. Concentrations are shown in μM. C. Oil red O-stained image of lipid-loaded hepatocytes simulated with CY or PPAR agonists. **D**. DiI-stained images of lipid-loaded cells incubated with CY or PPAR agonists. *, 1, untreated HepG2 cells; 2, lipid-loaded HepG2 cells; 3–7, lipid-loaded cells stimulated with CY (1, 5, 10, 50, or 100 μM, respectively); 8, lipid-loaded cells with 10 μM FF; 9, lipid-loaded cells with 1 μM GW0742; 10, lipid-loaded cells with 10 μM TT.

the position of the AF-2 domain after cyanidin binding, and may thus result in differential agonistic effects on PPARα, PPARδ/β, and PPARγ.

We initially screened ~900 Korean natural extracts and compounds and found that cyanidin had potent PPAR activity. Cyanidin and its glycosides are common in human foods, suggesting that we ingest significant quantities of these compounds each day [35]. Cyanidin has antioxidant activity, inhibiting LDL oxidation *in vitro* and *in vivo* [36–38], which is the basis of its cardioprotective effect.

Among the several possible applications of cyanidin as a PPAR modulator, hepatic steatosis and nonalcoholic fatty liver disease (NAFLD) are of interest. NAFLD is the clinical hepatic expression of metabolic syndrome, and has become the most common cause of liver disease worldwide [39]. PPARα activation in hepatocytes is known to favor normal lipid levels by reducing cellular TG concentration through modulation of target gene expression [40]. PPARγ ligands such as thiazolidinediones are also suggested that induce AMP-activated protein kinase expression, which increases fatty acid oxidation and decreases lipogenesis, decreases aminotransferase levels, and improves liver histology in patients with NAFLD [41]. Thus, we examined the effect of cyanidin on hepatic lipid accumulation. Intracellular cholesterol and TG levels were more effectively reduced by cyanidin compared to a full PPARα agonist. These hypolipidemic effects of cyanidin on lipid-loaded hepatocytes may be achieved primarily *via* the activation of PPARα through direct interaction and combined activation of the three PPAR subtypes.

The uptake of cyanidin is well known, although the specific protein transporters have not been clearly identified yet. Recent studies have demonstrated that the bioavailability of cyanidin and its glycosides is much higher than previously believed due to protein-mediated cellular uptake [42–46]. Multiple membrane transporters including bilitranslocase, MDR, and ABC transporters are suggested to be involved in flavonoid uptake by as-yet unidentified mechanisms and with unknown substrate specificities [47–49]. Transport of cyanidin-3-glucoside by bilitranslocase has been confirmed in the rodent vascular endothelium, and involvement of the MDR and ABC transporters has been suggested by uptake assays [48,50]; thus, *in vivo*, the cyanidin uptake process appears to be complex. However, one study suggested that uptake is exceptionally rapid *in vivo* due to protein-mediated cellular uptake, thus may provide the high transient bioavailability in spite of the apparent low plasma concentrations found in the studies [51].

Plasma cyanidin concentrations in the low micromolar range have been reported in several bioavailability studies in humans [52]. The EC_{50} of cyanidin for PPARα is about 3 μM, and thus we have suggested that the plasma cyanidin concentration activates PPARα. Although the plasma cyanidin concentration may be insufficient to induce PPARδ/β or γ transactivation, to our knowledge, there is no study of cyanidin bioavailability that has confirmed the cellular cyanidin concentrations in metabolically active tissues such as hepatocytes, muscle, and adipose tissue. In plant cells, anthocyanins, including cyanidin, are accumulated selectively at high concentrations in vacuoles. If a similar accumulation were to be demonstrated in human cells, it would be possible that despite its low plasma concentration, cyanidin could also activate PPARδ/β and γ, perhaps in the ER or peroxisomes. Therefore, we have suggested that cyanidin is a physiological agonist for PPARα and a potential ligand for PPARδ/β and γ.

Recent data have suggested that encapsulation with anthocyanin leads to a two-fold improvement in chemical stability [53,54]; thus, bioavailability studies of micro- or nano-encapsulation of cyanidin will be of great interest in terms of enhancing its bioactivity in human tissue. Intravenous injection of cyanidin represents an alternative approach

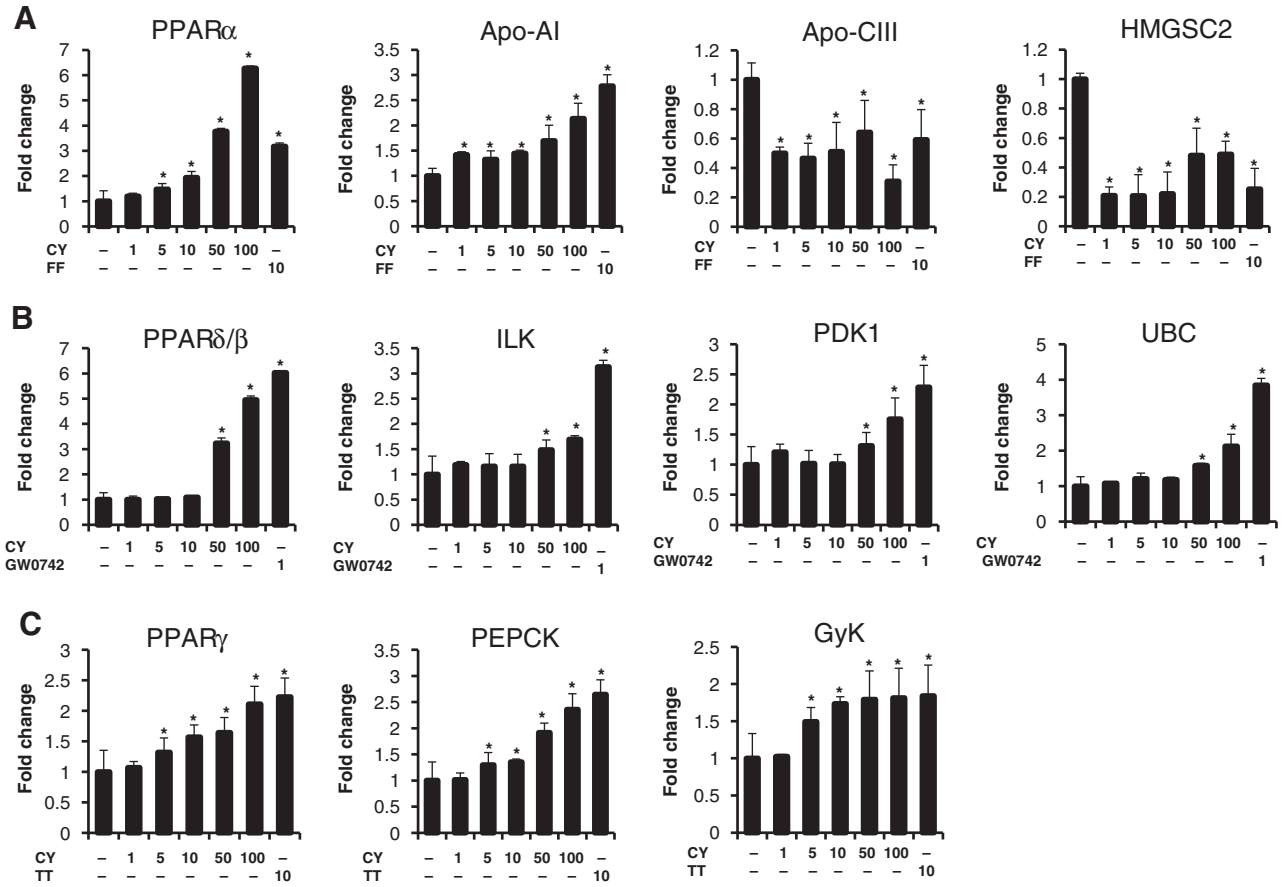


Fig. 6. Cyanidin (CY) regulates the expression of PPARs and their specific target genes. A. Expression of PPARα and its target genes. B. Expression of PPARδ/β and its target genes. C. Expression of PPARγ and its target genes. Results are normalized to the GAPDH mRNA level. *, $P<0.05$ and **, $P<0.001$ compared to controls. Concentrations are shown in μM.

for determining the effective cellular and plasma concentrations. Intravenous injection is being investigated in research on vitamin C to overcome bioavailability issues due to the tightly controlled intestinal uptake of vitamin C [55]. Thus, encapsulation and intravenous injection could overcome the low bioavailability of cyanidin.

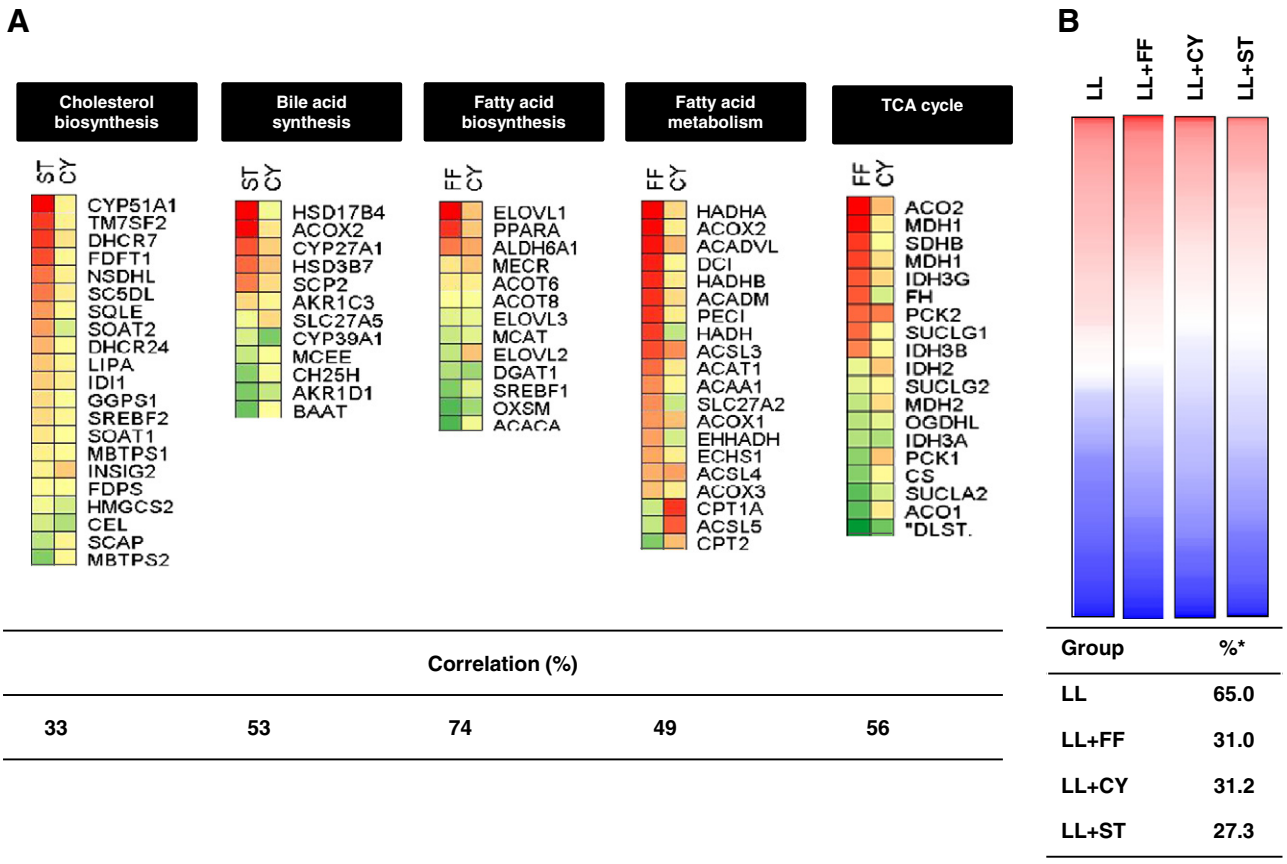
Cyanidin and its glycosides may be degraded into PCA and PGA [29]. PCA is reported to be a major metabolite in human [56], and the recent research has demonstrated the antiglycative and insulin-sensitizing effects of PCA *via* PPAR activation in both mice [29] and human primary cells [57]. Although some data suggest that the effects of PCA on luciferase activity are achieved by the activation of PPAR, there is no evidence of a direct interaction between PCA and PPAR LBD. Lin et al. [29] showed only altered PPARα and γ gene expression with PCA. Scazzocchino et al. [57] reported the results of qPCR and PPARγ activity. The PPARγ activity assay examines the binding of a PPARγ protein to the target promoter DNA sequence, and not a direct interaction between the ligand and transcription factor protein. Combined, the previous results do not suggest PCA binding PPAR proteins directly. In addition, our data clearly demonstrate that neither PCA nor PGA interacts directly with any of the three PPAR LBDs to induce co-activator recruitment. Thus we inferred that the effect of PCA was not *via* the direct binding of PCA and PPAR proteins but *via* other indirect pathways, potentially inducing endogenous ligand synthesis. However the unique target genes of each PPAR subtype and their transactivation detected in reporter gene assays in our experiments appear to be due to stimulation with cyanidin by direct binding.

Because cyanidin induced the activities of all three PPAR subtypes in a reporter gene assay, with the most potent effect on PPARα, we also investigated the global effects of cyanidin on the transcriptome of lipid-loaded hepatocytes. The data confirmed that cyanidin induced the expression of PPAR target genes. In hepatic transcriptome analysis, the expression profiles of 1231 genes that were commonly and significantly expressed in all experimental groups, including control hepatocytes, lipid-loaded hepatocytes, and lipid-loaded cells stimulated with cyanidin, fenofibrate, or lovastatin, showed that cyanidin rewired the expression of genes that were either up- or downregulated in lipid-loaded hepatocytes compared to untreated control cells. Moreover, the global effect of cyanidin (100 μM) was comparable to that of lovastatin (10 μM) and fenofibrate (10 μM).

We designed these experiments to compare the effects of cyanidin at a concentration 10 times that of either drug because the consumption of large quantities of cyanidin present in fruits and vegetables can result in blood concentrations of cyanidin that are much higher than that of either tested drug. We believe that our results are meaningful for the general public, *i.e.*, a high intake of cyanidin in fruits and vegetables may have biological effects comparable to those of hypolipidemic drugs. Collectively, these data suggest that cyanidin is a moderate ligand for PPARs; however, sufficient intake could result in significant biological effects if appropriate bioavailability could be guaranteed.

4.2. Conclusion

In conclusion, we suggest that cyanidin is a ligand of PPARα, δ/β, and γ, and a physiological agonist at least for PPARα and potentially for PPARδ/β and γ. According to its combined effects on these three PPAR subtypes, but primarily on PPARα, in hepatocytes, we suggest that cyanidin mediates lipid metabolism and mimics the effects of lovastatin and fenofibrate, leading to reduced cellular TG and cholesterol concentrations. Hence, cyanidin could be used as a natural compound to prevent NAFLD or assist in its treatment. Finally, the chemical structure of



Correlation (%)				
33	53	74	49	56

Group	%*
LL	65.0
LL+FF	31.0
LL+CY	31.2
LL+ST	27.3

Fig. 7. Cyanidin regulates the expression of PPAR-responsive genes in the cellular lipid and glucose metabolism pathways and normalizes the transcriptome profile induced after lipid loading in hepatocytes. A. The expression of genes involved in cholesterol and fatty acid metabolism and the tricarboxylic acid cycle. Red indicates induction of gene expression in the treatment group compared to the control, green indicates reduced expression, and yellow shows unaltered expression. B. Lipid-loaded hepatocytes were stimulated with cyanidin, fenofibrate, or lovastatin. Significantly upregulated (red) or downregulated (blue) expression between lipid-loaded hepatocytes and cyanidin-, lovavastatin-, or fenofibrated-treated lipid-loaded hepatocytes was represented. The middle part of the white portion indicates unchanged genes. *Percentage of selected genes with an expression level >20% of that of the non-treated control.

cyanidin may be applied to the development of potent PPAR dual- or pan-agonists.

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The authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2012.11.012.

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