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RESEARCH ARTICLE

The natural carotenoid astaxanthin, a PPAR-α agonist and PPAR-γ antagonist, reduces hepatic lipid accumulation by rewiring the transcriptome in lipid-loaded hepatocytes

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Scope: A natural carotenoid abundant in seafood, astaxanthin (AX), has hypolipidemic activity, but its underlying mechanisms of action and protein targets are unknown. We investigated the molecular mechanism of action of AX in hepatic hyperlipidemia by measuring peroxisome proliferator-activated receptors (PPAR) activity.

Methods and results: We examined the binding of AX to PPAR subtypes and its effects on hepatic lipid metabolism. AX binding activated PPAR-α, but inhibited PPAR-γ transactivation activity in reporter gene assay and time-resolved fluorescence energy transfer analyses. AX had no effect on PPARδ/β transactivation. AX bound directly to PPAR-α and PPAR-γ with moderate affinity, as assessed by surface plasmon resonance experiments. The differential effects of AX on PPARs were confirmed by measuring the expression of unique responsive genes for each PPAR subtype. AX significantly reduced cellular lipid accumulation in lipid-loaded hepatocytes. Transcriptome analysis revealed that the net effects of stimulation with AX (100 μM) on lipid metabolic pathways were similar to those elicited by fenofibrate and lovastatin (10 μM each), with AX rewiring the expression of genes involved in lipid metabolic pathways.

Conclusion: AX is a PPAR-α agonist and PPAR-γ antagonist, reduces hepatic lipid accumulation by rewiring the transcriptome in lipid-loaded hepatocytes.

Keywords:
Astaxanthin / Lipid metabolism / PPAR / Transcriptome / Seafood

1 Introduction

Peroxisome proliferator-activated receptors (PPARs) are well-characterized nuclear receptors that regulate the expression of genes involved in multiple biological pathways including cellular metabolic pathways, inflammation, differentiation, and proliferation [1]. PPAR proteins contain an N-terminal DNA-binding domain and a C-terminal ligand-binding domain (LBD) with a large ligand-binding pocket that interacts with natural and synthetic compounds [2]. The ligand-bound protein forms heterodimers with retinoid-X-receptors and other regulatory proteins. These heterodimers then activate or repress the transcription of target genes involved in various biological processes. A physiological role of PPARs is to regulate lipid and glucose metabolism. Appropriate regulation of PPAR activity could therefore provide therapeutic and preventive benefits in cardiovascular disease, type 2 diabetes mellitus (T2DM), and obesity [3–6].

The three subtypes of PPAR–alpha (α), delta/beta (δ/β), and gamma (γ)–have different metabolic functions [3, 7]. Briefly, PPAR-α is highly expressed in the liver, heart, and

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Abbreviations: **AX**, astaxanthin; **LBD**, ligand-binding domain; **PPAR**, peroxisome proliferator-activated receptors; **SPR**, surface plasmon resonance; **TCA**, tricarboxylic acid; **TG**, triglyceride; **TR-FRET**, time-resolved fluorescence resonance energy transfer; **T2DM**, type 2 diabetes mellitus; **TZDs**, thiazolidinediones

skeletal muscle and stimulates human fatty acid catabolism by accelerating the β-oxidation of fatty acids and increasing fatty acid uptake, thereby producing hypotriglyceridemic effects [8]. PPAR-α agonists known as fibrates reduce plasma triglyceride (TG) levels and increase high-density lipoprotein cholesterol (HDL-C) levels, thereby contributing to the treatment and prevention of atherosclerosis and cardiovascular events. Dysregulation of PPARδ/β could be involved in the pathophysiology of atherosclerosis and obesity due to its pivotal role in lipid metabolism, while the integrated activation of PPARδ/β, which acts to connect muscle and adipose tissue, is believed to lead to induction of nonshivering thermogenesis, thereby reducing fat accumulation in adipocytes and controlling body fat reduction [9]. PPAR-γ agonists including thiazolidinediones (TZDs) are widely prescribed as diabetes drugs since the activation of PPAR-γ improves insulin sensitivity and ameliorates insulin resistance. Overall, PPAR activation provides metabolic benefits. However, potent full agonists could induce significant side effects. For example, the potent TZD rosiglitazone induces carcinogenesis, edema, and cardiovascular complications, and has thus been suspended for newly diagnosed T2DM since 2010 [10]. In addition, recent focus in this area has centered on the concept of selective PPAR modulators–particularly for PPAR-γ–following the observation that the efficacies of full agonists, such as rosiglitazone and pioglitazone, were counterbalanced by receptor-mediated side effects including weight gain, edema, and potential cardiovascular complications [11]. A partial agonist might retain efficacy but reduce the transcriptional effects thought to be responsible for the attendant side effects. On the other hand, PPAR-γ antagonists such as GW9662 inhibit gene expression in adipocytes and adipocyte differentiation, and are thus considered to maximize the antiobesity effects [12]. Recent data also suggest that the PPAR-γ antagonist reduces lipid accumulation in steatotic livers [13].

Initially, we screened approximately 900 Korean natural extracts and compounds from plant and marine organisms for PPAR agonist or antagonist activity in a reporter gene assay-based screen, and found that astaxanthin (AX) had potent PPAR activity. AX is a naturally occurring carotenoid that is found in a wide variety of living organisms, including microalgae, yeast, salmon, and most crustaceans, including krill, shrimp, crawfish, crabs, and lobster, that are pink to red in color. As a carotenoid, AX is similar to beta-carotene in structure, but is not converted to vitamin A (retinol) in the human body, and thus has lower potential toxicity than other provitamin A carotenoids [14, 15].

AX is a powerful biological antioxidant that exhibits strong free radical scavenging activity [16–18], protecting against lipid peroxidation and oxidative damage of low-density lipoprotein cholesterol, cell membranes, cells, and tissues, and could provide antiatherogenic effects in animal models of cardiovascular disease [14, 19, 20]. Several animal studies of AX have been published. Hussein et al. suggested that AX administration increased the HDL-C level and lowered nonesterified fatty acid and TG levels in their animal model [21]. Also, AX-rich extract from green algae for four weeks in apoE-deficient mice significantly reduced plasma TG levels [22]. In another study, both hepatic and plasma TG concentrations and plasma cholesterol levels were significantly reduced in the AX supplementation group compared to controls [23]. Although several in vivo studies have suggested hypolipidemic effects of AX, the direct molecular target of AX has not been identified. The objective of this study is to investigated the molecular mechanism of action of AX in hepatic hyperlipidemia by measuring PPAR activity.

2 Materials and methods

Refer to the Supporting Information for detailed descriptions of the methods and supplementary data.

2.1 Reagents

Cell culture reagents and supplies were obtained from Hyclone (Logan, UT). AX (Fig. 1A), fenofibrate, troglitazone, GW9662, lovastatin were purchased from Sigma (St. Louis, MO). GW0742 was acquired from Cayman Chemical Company (Ann Arbor, MI). Total RNA extraction reagent (RNAiso Plus) and real-time polymerase chain reaction (RT-PCR) premix (SYBR® Premix Ex Taq™) were obtained from Takara (Otsu, Japan). Oligo(dT)$_{15}$ primer was purchased from Promega (Madison, WI). PowerOpti-ECL Western blotting detection reagent was purchased from Amersham-Pharmacia (Seoul, Korea). Primary (anti-PPARα, δ/β, γ, andtubulin) and secondary (antirabbit immunoglobulin G) antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Cell culture

HepG2 cells and Chinese hamster ovary (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) and Dulbecco's modified Eagle's medium-F/12 (DMEM-F/12; Hyclone), respectively, containing 10% heat-inactivated fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Welgene Inc., Seoul, Korea). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 as previously described [24].

2.3 Transfection and luciferase assays

Transfection and reporter gene assays were performed using CHO-K1 cells as described previously [25]. CHO-K1 cells were seeded in DMEM-F/12 in 24-well plates at a cell density of 2×10^5/well. The cells were then cotransfected with pSV-β-galactosidase, pSG5-PPAR alpha, and pBABE-zeo-PPAR

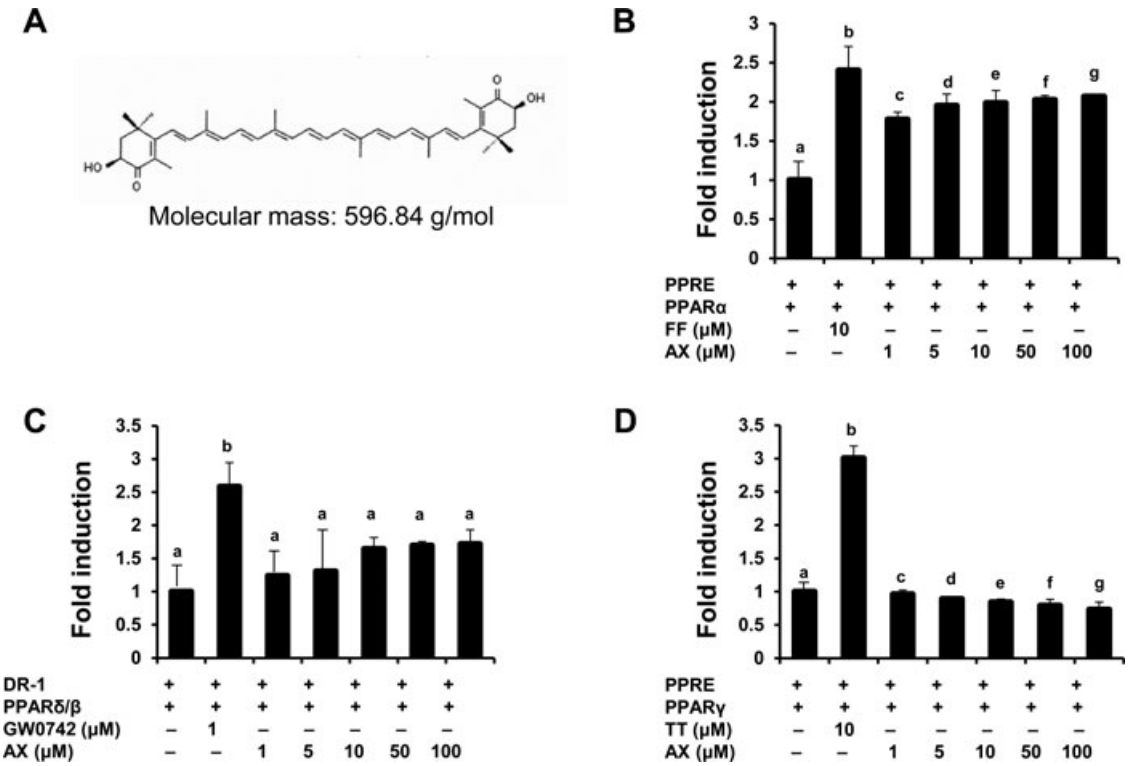

Figure 1. Astaxanthin (AX) activates PPAR-α and PPARδ/β, but inhibits PPAR-γ, as determined by a luciferase assay. (A) Chemical structure of AX. (B–D) Transactivation activities of PPAR subtypes induced by AX and agonists of PPAR subtypes. Fenofibrate (FF) for PPAR-α, GW0742 for PPARδ/β, and troglitazone (TT) for PPAR-γ were used as positive controls. Data represent the relative fold increase compared to the nontreated control (1% DMSO). Each analysis was performed in triplicate. All data were analyzed by using a one-way ANOVA for repeated measures. $p < 0.05$ indicates AX-treated hepatocytes compared to significant difference compared to control (PPRE/PPAR-α, DR-1/PPARδ/β, or PPRE/PPAR-γ) hepatocytes. A common letter means significant difference ($p < 0.05$).

gamma2 (Addgene, Cambridge, MA) with pCMV-3xPPRE-Luc, and pAdTrack-CMV-PPARδ/β (Addgene) with pCMV-DR-1, respectively. Transfection was performed with Hilymax (Dojindo, Gaithersburg, MD) according to the manufacturer's protocol. At 24 h posttransfection, AX was applied at different concentrations ranging from 1 to 100 μM in 1% DMSO. Next, the cells were lysed and assayed for luciferase and β-galactosidase activities using a firefly luciferase assay kit (Biotium, Hayward, CA) and a β-galactosidase enzyme assay system (Promega) according to the manufacturers' protocols. In each experiment, luciferase activity was normalized to β-galactosidase activity and expressed as relative luciferase activity.

2.4 Cloning, expression, and purification of PPAR proteins

The LBDs of human PPAR-α, PPARδ/β, and PPAR-γ were cloned into the expression vector pET-32 a-c(+) (Novagen, Madison, WI) to induce protein expression. Proteins were purified using a HiTrap™ Chelating HP Column (GE Healthcare, Milwaukee, WI) according to the manufacturer's instructions.

2.5 Surface plasmon resonance (SPR)

The binding of immobilized hPPARα-LBD, hPPARδ/β-LBD, and hPPARγ-LBD to ligands was measured using a Biacore 2000 instrument (HP/GE Healthcare) as described in Supporting Information.

2.6 Time-resolved fluorescence resonance energy transfer (TR-FRET) assays

The potential active or inhibitory effect of AX on PPARs was investigated using LanthaScreen™ TR-FRET PPAR-α, PPARδ/β, and PPAR-γ coactivator assays (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. This assay examined whether the ligand binding to PPAR-LBD protein induces coactivator recruitment capability.

2.7 Oil Red O staining of hepatocytes and intracellular cholesterol and TG measurement

Oil Red O staining of HepG2 cells and the extraction of cellular lipids were performed as described previously [26]. The cellular contents of cholesterol and TG were quantified using an enzymatic method with a Cobas C111 automatic analyzer (Roche, Basel, Switzerland).

2.8 Real-time quantitative PCR (qPCR) and protein immunoblotting analysis

Total RNA was extracted from HepG2 cells as described previously [27] after treatment with different concentrations of AX or fenofibrate (10 μM), GW0742 (1 μM), and troglitazone (10 μM) for 24 h. DMSO (1%) was used as a vehicle. Real-time qPCR was performed with Bio-Rad iQ SYBR® Green Supermix reagent and the Bio-Rad iQ5 Cycler System (Bio-Rad, Hercules, CA). Expression levels were calculated according to the manufacturer's guidelines. For protein immunoblotting analysis, total protein was isolated by adding 200 μL of RIPA containing 1% protease inhibitor cocktail (Bio Basic Inc., Amherst, NY) to the treated HepG2 cells and the concentration was determined with a BCA Protein Assay Kit (Pierce Biotechnology, Cramlington, UK). SDS-PAGE and immunoblotting were performed as described previously [28].

2.9 Oligonucleotide microarray analysis

Cytosolic lipid droplet formation was induced in HepG2 cells as described previously [27]. Lipid- and nonlipid-loaded HepG2 cells were treated with 100 μM AX, 10 μM fenofibrate, or 10 μM lovastatin, with 1% DMSO as the vehicle. Two-color microarray experiments were performed with HepG2 cells treated with 1% DMSO as a control. The synthesized cDNA was dye-labeled and hybridized onto Human OneArray microarrays (Phanlanx Biotech Group, Hsinchu, Taiwan). Data analysis was conducted according to the manufacturer's guidelines.

2.10 Statistical analysis

Data are presented as the mean ± SEM. The student's *t*-test was used to compare means between two groups. Statistical differences among groups were calculated by one-way analysis of variance (ANOVA) in reporter gene assay. A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 AX is an agonist for PPAR-α, but an antagonist for PPAR-γ

In the reporter gene assay, AX potently activated PPAR-α transactivation by 1.98-, 2.02-, and 2.03-fold at concentrations of 10, 50, and 100 μM, respectively, compared to the known PPAR-α agonist fenofibrate (Fig. 1B). AX has no effect on PPARδ/β activation (Fig. 1C). However, AX inhibited PPAR-γ transactivation by 22% (50 μM) and 28% (100 μM) (Fig. 1D).

AX, fenofibric acid, and troglitazone were analyzed with the SPR-BIAcore system to assess the direct interaction between PPAR-LBD proteins and AX. AX was directly associated with PPAR-α and PPAR-γ (Fig. 2A and B). The K_D values, the concentration at which a compound dissociates from the immobilized protein after the association phase, for AX were 197 μM to PPAR-α and 11.9 μM to PPAR-γ (Table 1). Thus, AX bound directly to PPAR-α and PPAR-γ, respectively, showing the higher affinity for PPAR-γ than PPAR-α.

To further investigate the activity of AX toward the three PPAR subtypes, the LBDs of PPAR-α, PPARδ/β, and PPAR-γ were incubated with the corresponding coactivator peptide at different concentrations of AX (10^{-3} to 10^{7} nM), using the TR-FRET coactivator assays with the agonist assay mode (Fig. 3). In line with previous findings, AX activated PPAR-α thus functioned as an agonist (Fig. 3A). Moreover, the TR-FRET ratio increased in a sigmoidal manner with increasing concentrations of AX, and the half-maximal effective concentration (EC_{50}) values, which represent the ability of AX to activate PPAR-α was 3.9 μM (Table 2). However, AX had no effect PPARδ/β activity (Fig. 3B). On the other hand, AX reduced the TR-FRET ratio of PPAR-γ with increasing concentrations of AX (Fig. 3C). Thus, we further investigated AX and PPAR-γ using the TR-FRET coactivator assay in antagonist assay mode. AX inhibited PPAR-γ (Fig. 3D), and the half-maximal inhibitory concentration (IC_{50}) value of AX for PPAR-γ was 607.8 μM (Table 2). The results suggest that AX may function as an antagonist for PPAR-γ. Therefore, the binding studies of AX with the LBDs of three subtypes of PPAR showed different effects. AX was an agonist for PPAR-α and had no effect on PPARδ/β, and an antagonist for PPAR-γ.

3.2 AX reduces cellular lipid concentration in lipid-loaded hepatocytes

Three subtypes of PPAR are commonly involved in hepatic lipid metabolism. We stimulated lipid-loaded hepatocytes with different concentrations of AX, as well as with fenofibrate, GW0742, and troglitazone, and vehicle (1% DMSO). The lipid staining results showed that AX stimulation reduced lipid accumulation in HepG2 cells. The effects were comparable to those of the PPAR agonists fenofibrate, GW0742, and troglitazone (Supporting Information Fig. S1). In addition,

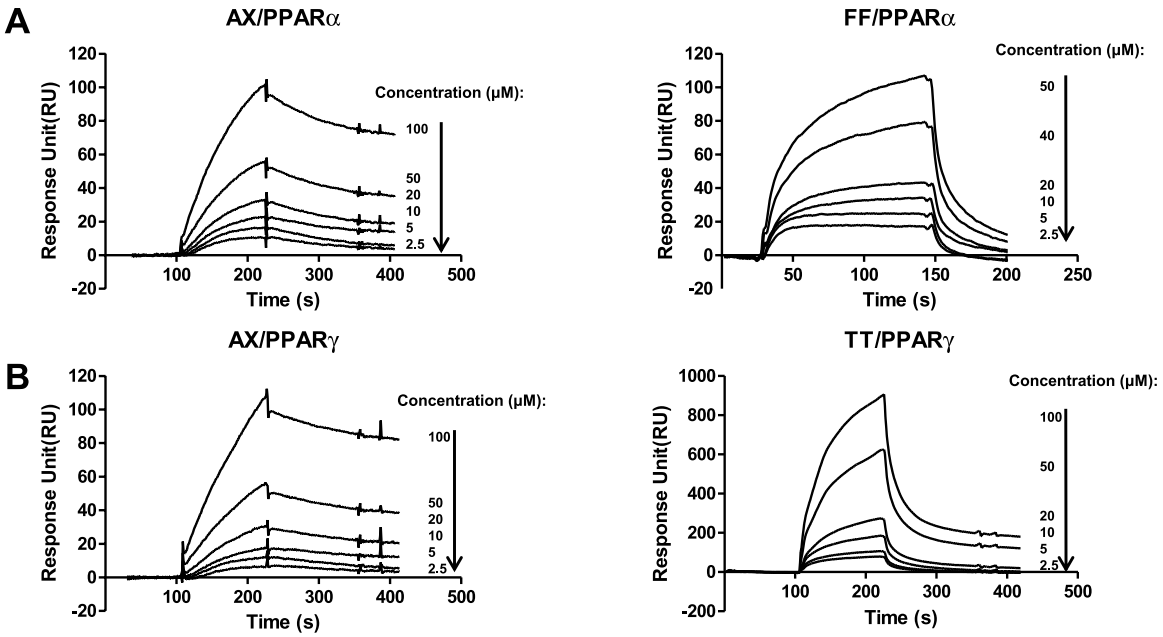

Figure 2. Astaxanthin (AX) binds directly to PPARα-LBD and PPARγ -LBD. The sensorgrams were obtained by injecting a series of concentrations of AX (left panels of A and B), fenofibrate (FF, right of A), or troglitazone (TT, right of C) over immobilized hPPARα-LBD or hPPARγ-LBD response in SPR-BIAcore as described in the methods and Supporting Information.

Table 1. Equilibrium dissociation constants (K_D values) for the binding of astaxanthin and PPAR agonists to hPPARα-LBD and hPPARγ-LBD, as measured by SPR

Compound	K_D (μM)	
	hPPARα-LBD	hPPARγ-LBD
Astaxanthin	197.0	11.9
Fenofibrate	0.3	–
Troglitazone	–	0.8

AX (10 μM) reduced the cellular cholesterol and TG concentrations in HepG2 cells, by −14 and −20%, respectively (Fig. 4A and B). These results confirmed that AX produces hypolipidemic effects in hepatocytes by regulating PPAR activity and suggest a potential application of AX for the prevention of nonalcoholic fatty liver disease.

3.3 AX induces PPARα-responsive gene expression, but reduces the expression of PPARγ-responsive genes

The expression of unique responsive genes for PPAR-α, PPARδ/β, and PPAR-γ was quantified in hepatocytes stimulated with AX. AX (5 μM) activated PPAR-α transcription 1.8-fold and upregulated the expression of the PPARα-responsive gene Apo-AI. Reduced expression of Apo-CIII and HMGCS2, which is mediated by PPAR-α activation, was demonstrated by AX stimulation (Fig. 5A). AX treatment did not significantly change the gene expression of PPARδ/β, or its target genes ILK, PDK1, or UBC (Fig. 5B), in line with the results of the TR-FRET assay of AX with PPARδ/β, indicating that the effect of AX on PPARδ/β is marginal. However, AX (10 μM) inhibited PPAR-γ gene expression by 28%. The PPARγ-responsive genes AQP7 and PEPCK were also significantly downregulated by AX (100 μM), by 30 and 20%, respectively (Fig. 5C).

3.4 Global effects of AX on the hepatic transcriptome in lipid-loaded HepG2 cells

The genome-wide hypolipidemic effects of AX (100 μM) in hepatocytes were compared to those of two lipid-lowering drugs, lovastatin (10 μM) and fenofibrate (10 μM), in lipid-loaded hepatocytes (Fig. 6). AX upregulated the gene expression of PPAR-α 2.14-fold but downregulated PPAR-γ expression 0.67-fold in microarray. AX stimulated the bile acid synthesis pathway and inhibited the cholesterol biosynthesis pathway, comparable to the effects of lovastatin. Expression of genes involved in fatty acid biosynthesis, fatty acid metabolism, the glycolysis pathway, and the tricarboxylic acid (TCA) cycle was similarly altered in cells stimulated with AX (100 μM) and those treated with fenofibrate (10 μM), suggesting that AX may regulate hepatic fatty acid metabolism gene expression in a manner comparable to fenofibrate. Furthermore, the expression of target genes for PPAR-α and PPAR-γ was significantly changed: SCP2, ACADVL, ACADM, and EHHADH were induced, whereas CPT2 and ACO1 were

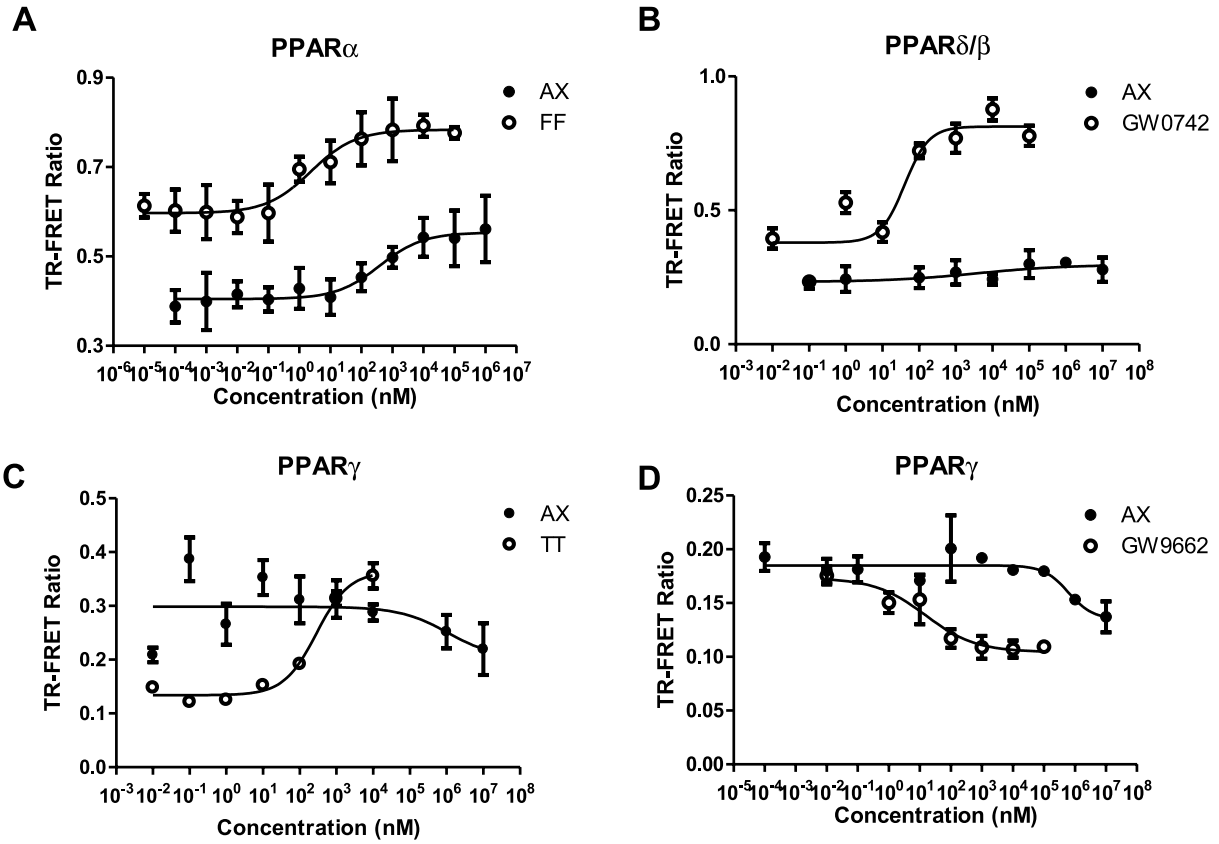

Figure 3. Astaxanthin (AX) binding activates PPAR-α and PPARδ/β, but inhibits PPAR-γ, as determined by a TR-FRET assay. Increasing concentrations of AX or PPAR agonists were added to LBD and coactivator peptide solutions. (A) AX (left plot) and fenofibrate (FF, right plot) activate PPAR-α; (B) AX (left) and GW0742 (right) activate PPARδ/β; (C) AX (left) inhibits PPAR-γ, whereas troglitazone (TT, right) activates PPAR-γ; (D) AX (left) and GW9662 (right) inhibit PPAR-γ.

Table 2. Half-maximal effective concentrations (EC_{50} values) of astaxanthin and PPAR agonists for hPPARα-LBD and half-maximal inhibitory concentrations (IC_{50} values) of astaxanthin and PPARγ antagonists for hPPARγ-LBD, as measured by a TR-FRET assay

Compound	EC_{50} for hPPARα-LBD	IC_{50} for hPPARγ-LBD
Astaxanthin	3.9 μM	607.8 μM
Fenofibrate	446.3 nM	–
GW9662	–	14.9 nM

reduced. The common target gene (CYP27A1), which is up-regulated by both PPAR-α and PPAR-γ activation, was induced significantly, suggesting a dominant effect of PPAR-α over the γ-form on CYP27A1 gene expression (Fig. 6A). These microarray results suggest that AX could modulate PPAR-α and PPAR-γ. A list of the genes included in these pathways and their expressions are provided in Supporting Information Table S1.

To confirm the microarray results, we selected 12 genes involved in lipid and glucose metabolism and quantified their expression by qPCR. The expression of these genes was comparable between the qPCR and microarray results (Supporting Information Fig. S2).

3.5 AX stimulation of lipid-loaded HepG2 cells rewired the hepatic transcriptome profile, with the effects of AX being comparable to those of lovastatin and fenofibrate

We selected 388 genes that were commonly and significantly expressed in all experimental groups, including control hepatocytes, lipid-loaded hepatocytes, and lipid-loaded cells stimulated with AX, fenofibrate, or lovastatin, respectively, to compare the transcriptome profile of AX with those of hypolipidemic drugs. Lipid loading of hepatocytes dramatically altered hepatic gene expression, where 74.2% of the selected genes showed >20% changes in expression [fold change >1.2 (red color) or fold change <0.8 (blue color)]. However, AX stimulation of lipid-loaded cells showed that 55.7% of the selected genes had expression changes of >20%. Thus, 18.5% of the selected genes became to show less than 20% changes in expression after AX stimulation compared to the

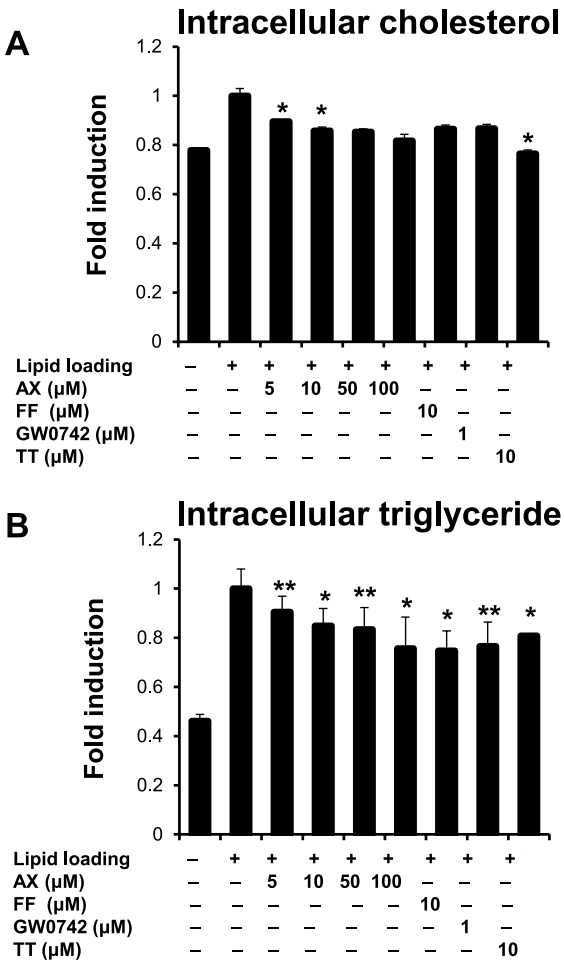

Figure 4. Astaxanthin (AX) reduces intracellular cholesterol and triglyceride contents in lipid-loaded hepatocytes. HepG2 cells were incubated with palmitic acid (400 μM) and oleic acid (400 μM) for 24 h, and then stimulated with AX (5–100 μM), fenofibrate (FF, 10 μM), GW0742 (1 μM), or troglitazone (TT, 10 μM). (A) Cellular cholesterol content. (B) Cellular triglyceride content (changes in cholesterol and triglyceride contents of hepatocytes treated with fatty acids were considered a single fold change. All data were analyzed by one-way ANOVA for repeated measures. $*p < 0.05$, $**p < 0.001$. AX-, fenofibrate-, GW0742-, or troglitazone-treated hepatocytes compared to lipid-loaded sample and control hepatocytes.

lipid-loaded condition. Fenofibrate and lovastatin stimulation, respectively, showed 57.2 and 46.4% of the selected gene expression changes of >20%. Thus, 17.0 and 27.8% of the selected genes fell into the <20% change category compared to the lipid-loaded condition after fenofibrate and lovastatin stimulation, respectively (Fig. 6B). Lovastatin had the most potent normalizing effect on gene expression (<20% change compared to the control) in lipid-loaded hepatocytes. The results indicate that the effects of dietary intake of AX may be comparable to those of lovastatin and fenofibrate for mediating the hepatic transcriptome profile.

4 Discussion

PPARs belong to a superfamily of nuclear receptors. Ligand-induced activation of PPARs controls the expression of innumerable genes, notably genes involved in lipid and lipoprotein metabolism whose activation could prevent or ameliorate clinical symptoms in hyperlipidemia, insulin resistance, and obesity, and that have thus been favorable targets for drug development as well as nutritional interventions with natural compounds and phytonutrients [11]. Identification and characterization of natural or synthetic ligands specific for each PPAR isoform has been of great interest in research. However, known synthetic ligands specific for individual PPAR isoforms cause considerable side effects, e.g. the induction of carcinogenesis (PPAR-α) and increased risk of heart failure (PPAR-γ overactivation) [29]. Therefore, recent efforts have focused on the identification of a hit compound derived from natural compounds and phytonutrients with moderate binding affinity for two or more PPAR isoforms, so-called dual- or pan-PPAR ligands, for the development of balanced ligands [30, 31].

Previously, we screened approximately 900 natural compounds and extracts from plants and marine organisms for PPAR activity in a luciferase-based assay and identified AX as a compound with strong PPAR-modulating activity. In the current study, we demonstrated that AX, a natural carotenoid abundant in seafood, bound directly to PPAR-α and PPAR-γ isoforms with physiologically significant affinities, and that binding induced opposite effects on PPAR-α and PPAR-γ, which is unique. AX is a moderate agonist for PPAR-α, but an antagonist for PPAR-γ. Several synthetic and natural compounds have been identified as PPAR pan-agonists. These include the antihyperlipidemic drug bezafibrate [32], which is used to treat T2DM and cardiovascular diseases. Nonetheless, until now, no compounds were known that bound PPAR subtypes, inducing opposite effects. To the best of our knowledge, the current study is the first report of a natural compound that acts as an agonist for PPAR-α, but an antagonist for PPAR-γ. Our data also provide a structural insight into a PPAR-α agonist/PPAR-γ antagonist, and suggest that AX could serve as a scaffold for the synthesis of more effective compounds.

The results of structural studies suggest that the three PPAR subtypes share a similar general mechanism of activation, with the bound ligand stabilizing the C-terminal activating helix (AF-2) by making hydrophobic contacts with it, thereby allowing the binding of coactivators [33]. Although an agonist binding allows the AF-2 domain to adopt the active conformation, binding interactions between corepressors and the PPAR-LBD occur in the unliganded state and can be stabilized by antagonists [34]. Three-dimensional structural studies have revealed that compared to the bulkier Cys275 and Arg284 side chains in PPAR-α, PPAR-γ has a smaller Gly284 side chain in the ligand-binding pocket [33] and this difference may affect the position of the AF-2 domain after AX binding, and may thus result in opposite effects in

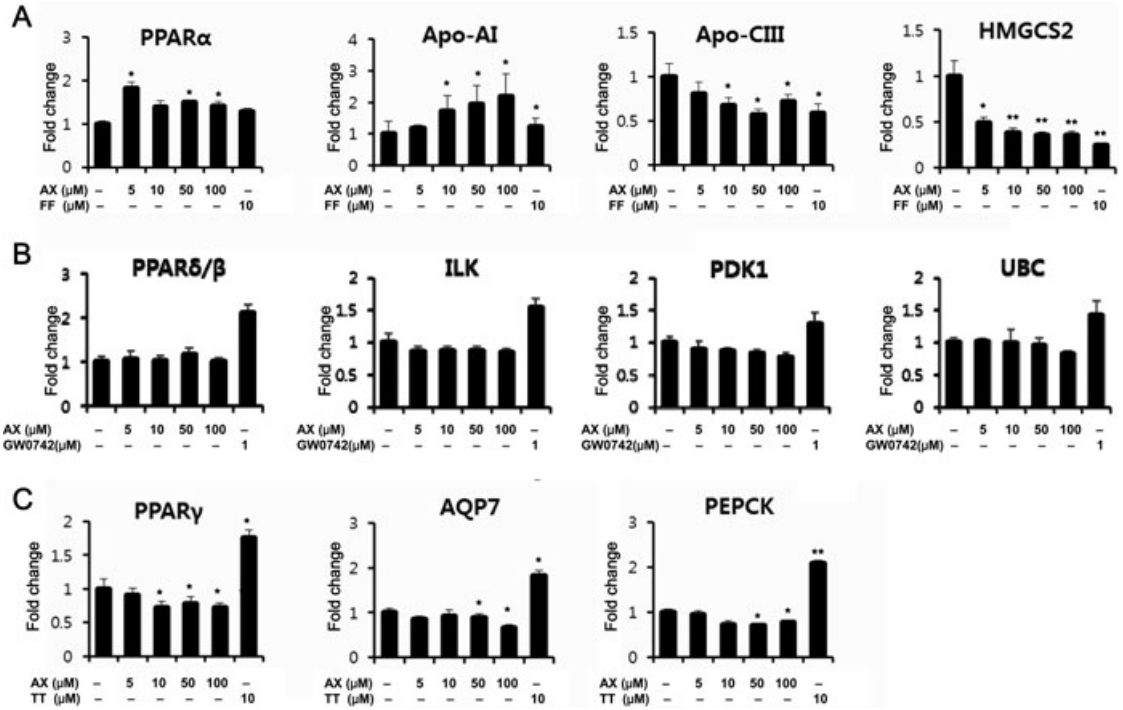

Figure 5. Astaxanthin (AX) changes the expression of PPARs and regulates transcription of their specific target genes. HepG2 cells were treated with AX (5–100 μM), fenofibrate (FF, 10 μM), GW0742 (1 μM), or troglitazone (TT, 10 μM) for 24 h, with DMSO (1%) as the vehicle, and the changes in gene expression were investigated. (A) Gene expression of PPAR-α and its target genes; (B) gene expression of PPARδ/β and its target genes; (C) gene expression of PPAR-γ and its target genes. Results are normalized to the GAPDH mRNA level. $*p < 0.05$, $**p < 0.001$ compared to the control. The full names of unique PPAR target genes are provided in Supporting Information Table S2.

PPAR-α and PPAR-γ. Ligand-binding cavity of the PPARδ/β is significantly narrower in the region adjacent to the AF2 helix, such that PPARδ/β may have stricter ligand specificity compared with PPAR-α and PPAR-γ, hardly accommodating bulky substituents of the ligand in the region that comes in contact with the LBD cavity [33]; thus, AX may not induce significant transactivation on PPARδ/β.

Several groups have described the in vivo lipid-regulating effects of AX. For example, oral administration of AX (50 mg/kg/day) for 22 weeks reduced nonesterified fatty acid and TG concentrations while elevating HDL-C levels in an animal model [21], while AX supplementation in Wistar rats with alloxan-induced diabetes for 23 days significantly reduced plasma TG levels [35]. In another study, hepatic and plasma TG concentrations and plasma cholesterol levels were significantly reduced in the AX supplementation group compared to the control [23]. In 2009, Yoshida reported the results of a randomized, placebo-controlled clinical trial with AX that showed a reduction in plasma TG levels and an increase in HDL-C in humans [36]. However, the direct molecular target of AX has not been identified. Our findings demonstrate that the target proteins for AX are PPAR-α and PPAR-γ, which reduce cellular and plasma TG concentrations by rewiring the hepatic transcriptome.

Since AX binding induced opposite effects on PPAR-α and PPAR-γ, we decided to investigate the global effects of AX on the hepatic transcriptome in lipid-loaded hepatocytes. The results confirmed that AX induced the expression of PPAR-α target genes while suppressing PPARγ-specific target genes. In addition, the expression profiles of 388 genes that were commonly and significantly expressed in all experimental groups, including control hepatocytes, lipid-loaded hepatocytes, and lipid-loaded cells stimulated with AX, fenofibrate, or lovastatin showed that AX rewired the expression of genes that were either up- or downregulated in lipid-loaded hepatocytes compared to untreated control cells, and that the global effect of AX (100 μM) was comparable to that of lovastatin (10 μM) or fenofibrate (10 μM). This indicates that AX is a weak ligand for PPARs but sufficient dietary intake could show significant biological effects.

The bioavailability of AX has been investigated in vitro and in vivo. Results from the Caco-2 cell-based Transwell system showed 14–27% of total uptake and secretion to the basolateral chamber—the highest percentage total uptake and secretion among seven tested carotenoids [37]. Human studies also showed significant uptake of AX and a study reported approximately 6% of the administered dose of AX appeared in the plasma after supplementation with 100 mg of AX [38]. Thus, intake of AX would enable it to function as a ligand for PPARs in human tissues.

Among several possible applications of AX as a dual PPAR-α and PPAR-γ modulator, hepatic steatosis and nonalcoholic

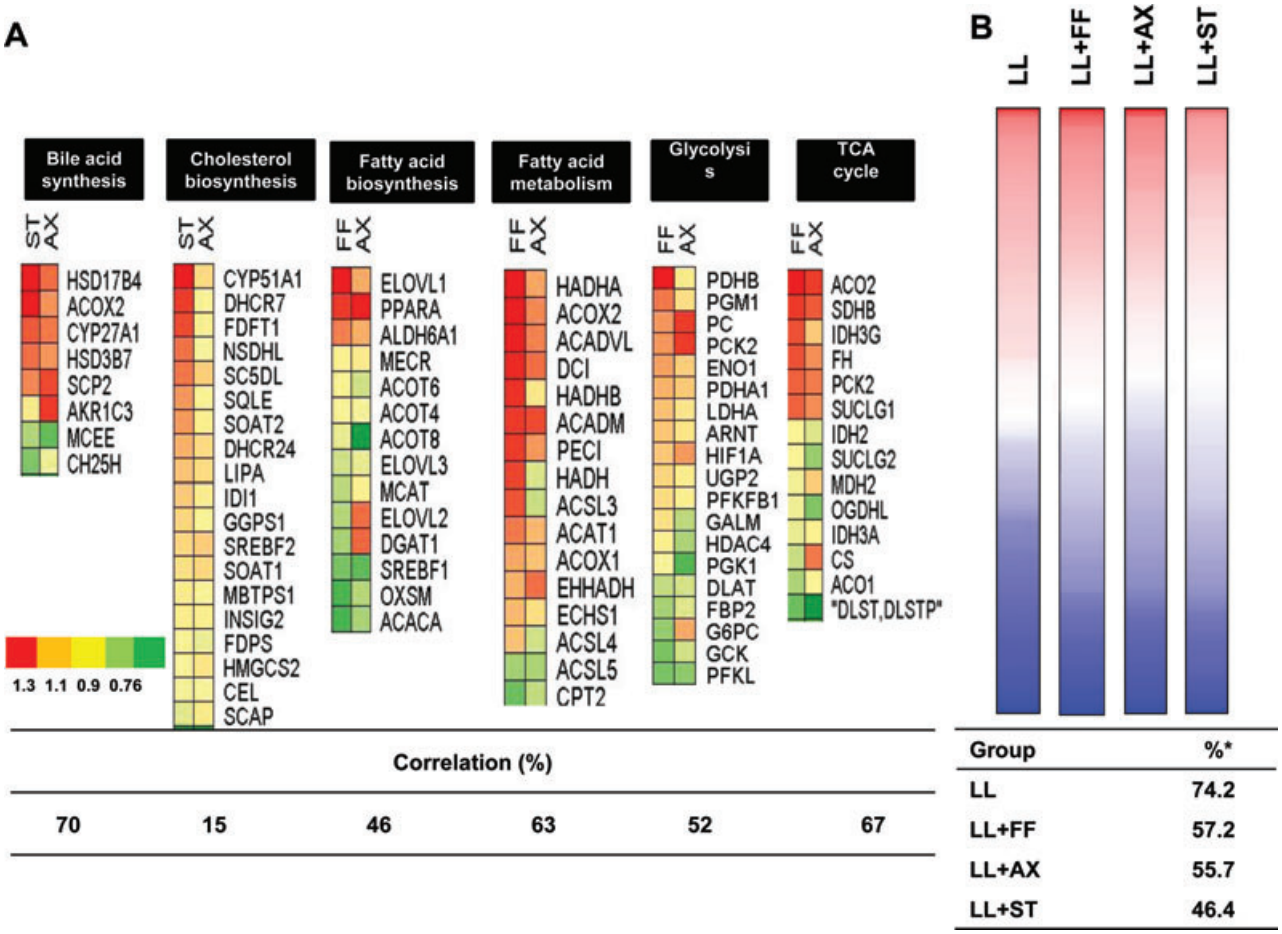

Figure 6. Astaxanthin (AX) regulates PPAR-responsive gene expression in cellular lipid and glucose metabolism pathways and normalizes the hepatic transcriptome profile induced after lipid loading in hepatocytes. HepG2 cells were treated with lipid for 24 h and the lipid was removed. Then, cells were treated with AX (100 μM), fenofibrate (FF, 10 μM), or lovastatin (ST, 10 μM) for the next 24 h. Control cells were treated with the vehicle (1% DMSO). (A) The gene expression in the bile acid synthesis pathway, cholesterol biosynthesis pathway, fatty acid biosynthesis pathway, fatty acid metabolism pathway, glycolysis pathway, and tricarboxylic acid (TCA) cycle pathway changed (altered expression is indicated by different colors). The expression of target genes of all subtypes of PPAR (PPAR-α, PPARδ/β, and PPAR-γ) increased (SCP2, ACADVL, ACADM, and EHHADH) or decreased (CPT2 and ACO1). The expression of the PPAR-α and PPAR-γ target gene CYP27A1 increased, while that of the PPAR-α target gene HMGCS2 decreased. The correlation indicates the similarity of gene expression in AX-stimulated hepatocytes and fenofibrate- or lovastatin-treated hepatocytes. (B) Lipid-loaded hepatocytes were treated with AX, lovastatin, or fenofibrate. Significantly upregulated (red) and downregulated (blue) expression compared between lipid-loaded hepatocytes and AX-, lovastatin-, or fenofibrate-treated lipid-loaded hepatocytes. The percentage of changed selected genes (presented as fold change 1 ± 0.2) by lipid-loaded cells or AX-, fenofibrate-, or lovastatin-treated cells was calculated. The middle part of the white portion indicates the unchanged selected genes. *Percentage of selected genes with >20% expression compared to the nontreated controls.

fatty liver disease are of interest. Nonalcoholic fatty liver disease is the clinical hepatic expression of metabolic syndrome. Its prevalence is approximately 20–30%, with a rapid increase in the metabolic risk factors in the general population, and it has become the most common cause of liver disease worldwide [39]. PPAR-α activation in hepatocytes is well known to favor normal lipid levels in hepatocytes by reducing cellular TG concentration through the modulation of target gene expression [40]. However, the role of PPAR-γ in hepatic steatosis has been controversial. Recently, Clària and colleagues demonstrated that PPAR-γ is a presteatotic factor in fatty liver and showed that targeted deletion of PPAR-γ in hepatocytes protected mice against high-fat diet-induced hepatic steatosis by downregulating the expression of genes involved in lipogenesis and lipid transport [13]. In hepatocytes, a PPAR-γ agonist and oleic acid increased TG accumulation, an effect that was blocked by a PPAR-γ antagonist [41]. Collectively, the current data suggest that PPAR-γ antagonists could ameliorate hepatic lipid accumulation. We thus examined the effect of AX, as a moderate PPAR-α agonist and a PPAR-γ antagonist, on hepatic lipid accumulation. Our results show that intracellular cholesterol and TG levels were more effectively reduced by AX compared to a full PPAR-α agonist, and that the hepatic transcriptome of lipid metabolism pathways in

lipid-loaded hepatocytes was normalized to levels in control hepatocytes by AX stimulation.

Additionally, our gene transcriptome profiling results suggest that AX regulates glucose metabolism genes and stimulates gluconeogenesis through PCK gene upregulation, in line with a previous report [15, 21]. AX also altered the expression of genes involved in both glycolysis and the TCA cycle to a similar manner to fenofibrate. These results suggest that the previously reported hypoglycemic and hypoinsulinemic effects of AX in animals [42] could be achieved through regulation of PPAR-α and PPAR-γ activity, respectively.

Regarding obesity prevention, research suggests the existence of an interesting paradox concerning PPAR-γ, with both activation of PPAR-γ by TZD drugs and inhibition of PPAR-γ by antagonists or gene deficiency protecting against obesity-induced insulin resistance in animal models [12]. Because TZDs, agonists of PPAR-γ, have caused significant side effects, the potential benefits of PPAR-γ antagonists in obesity treatment have recently gained attention. Thus, AX, which has PPAR-γ antagonist activity, may have potential in the nutritional prevention of obesity because it reduces plasma TG levels and simultaneously activates PPAR-α [4, 43].

In conclusion, we demonstrated that AX is an agonist of PPAR-α, but an antagonist of PPAR-γ, thus reduces hepatic lipid accumulation effectively. Stimulation of hepatocytes with AX rewires global hepatic lipid metabolism gene expression in a way that could mimic the effects of lovastatin and fenofibrate, thus our results suggest that the intake of seafood containing high concentrations of AX may be beneficial in the control of plasma lipid concentrations and hepatic steatosis. Our data also provide a structural insight into a PPAR-α agonist/PPAR-γ antagonist, and suggest that AX could serve as a scaffold for the synthesis of more effective compounds.

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5 References

[1] Bensinger, S. J., Tontonoz, P., Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature* 2008, *454*, 470–477.

[2] Nettles, K. W., Insights into PPAR gamma from structures with endogenous and covalently bound ligands. *Nat. Struct. Mol. Biol.* 2008, *15*, 893–895.

[3] van Bilsen, M., van Nieuwenhoven, F. A., PPARs as therapeutic targets in cardiovascular disease. *Expert Opin. Ther. Targets* 2010, *14*, 1029–1045.

[4] Robinson, E., Grieve, D. J., Significance of peroxisome proliferator-activated receptors in the cardiovascular system in health and disease. *Pharmacol. Ther.* 2009, *122*, 246–263.

[5] Pineda Torra, I., Gervois, P., Staels, B., Peroxisome proliferator-activated receptor alpha in metabolic disease, inflammation, atherosclerosis and aging. *Curr. Opin. Lipidol.* 1999, *10*, 151–159.

[6] McGarry, J. D., Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* 2002, *51*, 7–18.

[7] Latruffe, N., Vamecq, J., Peroxisome proliferators and peroxisome proliferator activated receptors (PPARs) as regulators of lipid metabolism. *Biochimie* 1997, *79*, 81–94.

[8] Neve, B. P., Fruchart, J. C., Staels, B., Role of the peroxisome proliferator-activated receptors (PPAR) in atherosclerosis. *Bioche. Pharmacol.* 2000, *60*, 1245–1250.

[9] Barish, G. D., Narkar, V. A., Evans, R. M., PPAR delta: a dagger in the heart of the metabolic syndrome. *J. Clin. Invest.* 2006, *116*, 590–597.

[10] Cohen, D., FDA puts rosiglitazone post-marketing trial on hold. *Br. Med. J.* 2010, *341*, c4017.

[11] Huang, T. H., Teoh, A. W., Lin, B. L., Lin, D. S., et al., The role of herbal PPAR modulators in the treatment of cardiometabolic syndrome. *Pharmacol. Res.* 2009, *60*, 195–206.

[12] Chinetti-Gbaguidi, G., Fruchart, J. C., Staels, B., Role of the PPAR family of nuclear receptors in the regulation of metabolic and cardiovascular homeostasis: new approaches to therapy. *Curr. Opin. Pharmacol.* 2005, *5*, 177–183.

[13] Moran-Salvador, E., Lopez-Parra, M., Garcia-Alonso, V., Titos, E., et al., Role for PPARgamma in obesity-induced hepatic steatosis as determined by hepatocyte- and macrophage-specific conditional knockouts. *FASEB J.* 2011, *25*, 2538–2550.

[14] Fassett, R. G., Coombes, J. S., Astaxanthin, oxidative stress, inflammation and cardiovascular disease. *Future Cardiol.* 2009, *5*, 333–342.

[15] Fassett, R. G., Coombes, J. S., Astaxanthin: a potential therapeutic agent in cardiovascular disease. *Mar. Drugs.* 2011, *9*, 447–465.

[16] Wolf, A. M., Asoh, S., Hiranuma, H., Ohsawa, I., et al., Astaxanthin protects mitochondrial redox state and functional integrity against oxidative stress. *J. Nutr. Biochem.* 2010, *21*, 381–389.

[17] Jacobsson, L. S., Yuan, X. M., Zieden, B., Olsson, A. G., Effects of alpha-tocopherol and astaxanthin on LDL oxidation and atherosclerosis in WHHL rabbits. *Atherosclerosis* 2004, *173*, 231–237.

[18] Iwamoto, T., Hosoda, K., Hirano, R., Kurata, H., et al., Inhibition of low-density lipoprotein oxidation by astaxanthin. *J. Atheroscler. Thromb.* 2000, *7*, 216–222.

[19] Pashkow, F. J., Watumull, D. G., Campbell, C. L., Astaxanthin: a novel potential treatment for oxidative stress and

inflammation in cardiovascular disease. *Am. J. Cardiol.* 2008, *101*, 58d–68d.

[20] Sevilla, M. A., Monroy-Ruiz, J., Carron, R., Montero, M. J., Astaxanthin-enriched-diet reduces blood pressure and improves cardiovascular parameters in spontaneously hypertensive rats. *Pharmacol. Res.* 2011, *63*, 44–50.

[21] Hussein, G., Nakagawa, T., Goto, H., Shimada, Y., et al., Astaxanthin ameliorates features of metabolic syndrome in SHR/NDmcr-cp. *Life Sci.* 2007, *80*, 522–529.

[22] Yang, Y., Seo, J. M., Nguyen, A., Pham, T. X., et al., Astaxanthin-rich extract from the green alga *Haematococcus pluvialis* lowers plasma lipid concentrations and enhances antioxidant defense in apolipoprotein E knockout mice. *J. Nutr.* 2011, *141*, 1611–1617.

[23] Yazawa, K., Ikeuchi, M., Koyama, T., Takahashi, J., Effects of astaxanthin in obese mice fed a high-fat diet. *Biosci. Biotechnol. Biochem.* 2007, *71*, 893–899.

[24] Jun, H.-J., Chung, M., Dawson, K., Rodriguez, R., et al., Nutrigenomic analysis of hypolipidemic effects of *Agastache rugosa* essential oils in HepG2 cells and C57 BL/6 mice. *Food Sci. Biotechnol.* 2010, *19*, 219–227.

[25] Jia, Y., Bhuiyan, M. J., Jun, H. J., Lee, J. H., et al., Ursolic acid is a PPAR-alpha agonist that regulates hepatic lipid metabolism. *Bioorg. Med. Chem. Lett.* 2011, *21*, 5876–5880.

[26] Hoang, M. H., Houng, S. J., Jun, H. J., Lee, J. H., et al., Barley intake induces bile acid excretion by reduced expression of intestinal ASBT and NPC1L1 in C57 BL/6 J mice. *J. Agric. Food Chem.* 2011, *59*, 6798–6805.

[27] Bhuiyan, M. J., Do, H. V., Mun, S., Jun, H. J., et al., Hypocholesterolemic and hypoglycemic effects of enzymatically modified carbohydrates from rice in high-fat-fed C57 BL/6 J mice. *Mol. Nutr. Food. Res.* 2011, *55*(Suppl 2), S214–S226.

[28] Park, H. M., Lee, J. H., Yaoyao, J., Jun, H. J., et al., Limonene, a natural cyclic terpene, is an agonistic ligand for adenosine A(2 A) receptors. *Biochem. Biophys. Res. Commun.* 2011, *404*, 345–348.

[29] Staels, B., Rubenstrunk, A., Hanf, R., Hum, D. W., et al., Safety issues and prospects for future generations of PPAR modulators. *Biochim. Biophys. Acta-Mol. Cell Biol.* 2007, *1771*, 1065–1081.

[30] Balakumar, P., Rose, M., Ganti, S. S., Krishan, P., et al., PPAR dual agonists: are they opening Pandora's box? *Pharmacol. Res.* 2007, *56*, 91–98.

[31] Fievet, C., Fruchart, J. C., Staels, B., PPARalpha and PPARgamma dual agonists for the treatment of type 2 diabetes and the metabolic syndrome. *Curr. Opin. Pharmacol.* 2006, *6*, 606–614.

[32] Nagasawa, T., Inada, Y., Nakano, S., Tamura, T., et al., Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARdelta agonist, on development of steatohepatitis in mice fed a methionine- and choline-deficient diet. *Eur. J. Pharmacol.* 2006, *536*, 182–191.

[33] Zoete, V., Grosdidier, A., Michielin, O., Peroxisome proliferator-activated receptor structures: ligand specificity, molecular switch and interactions with regulators. *Biochim. Biophys. Acta* 2007, *1771*, 915–925.

[34] Xu, H. E., Stanley, T. B., Montana, V. G., Lambert, M. H., et al., Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARalpha. *Nature* 2002, *415*, 813–817.

[35] de Barros, M. P., Otton, R., Marin, D. P., Bolin, A. P., et al., Astaxanthin ameliorates the redox imbalance in lymphocytes of experimental diabetic rats. *Chem. Biol. Interact.* 2010, *186*, 306–315.

[36] Yoshida, H., Yanai, H., Ito, K., Tomono, Y., et al., Administration of natural astaxanthin increases serum HDL-cholesterol and adiponectin in subjects with mild hyperlipidemia. *Atherosclerosis* 2010, *209*, 520–523.

[37] O'Sullivan, L., Ryan, L., O'Brien, N., Comparison of the uptake and secretion of carotene and xanthophyll carotenoids by Caco-2 intestinal cells. *Br. J. Nutr.* 2007, *98*, 38–44.

[38] Osterlie, M., Bjerkeng, B., Liaaen-Jensen, S., Plasma appearance and distribution of astaxanthin E/Z and R/S isomers in plasma lipoproteins of men after single dose administration of astaxanthin. *J. Nutr. Biochem.* 2000, *11*, 482–490.

[39] Postic, C., Girard, J., Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J. Clin. Invest.* 2008, *118*, 829–838.

[40] Lefebvre, P., Chinetti, G., Fruchart, J. C., Staels, B., Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis. *J. Clin. Invest.* 2006, *116*, 571–580.

[41] Suzuki, K., Takahashi, K., Nishimaki-Mogami, T., Kagechika, H., et al., Docosahexaenoic acid induces adipose differentiation-related protein through activation of retinoid x receptor in human choriocarcinoma BeWo cells. *Biol. Pharm. Bull.* 2009, *32*, 1177–1182.

[42] Preuss, H. G., Echard, B., Yamashita, E., Perricone, N. V., High dose astaxanthin lowers blood pressure and increases insulin sensitivity in rats: are these effects interdependent? *Inter. J. Med. Sci.* 2011, *8*, 126–138.

[43] Le May, C., Pineau, T., Bigot, K., Kohl, C., et al., Reduced hepatic fatty acid oxidation in fasting PPARalpha null mice is due to impaired mitochondrial hydroxymethylglutaryl-CoA synthase gene expression. *FEBS Lett.* 2000, *475*, 163–166.

