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Astaxanthin reduces hepatic lipid accumulations in high-fat-fed C57BL/6J mice via activation of peroxisome proliferator-activated receptor (PPAR) alpha and inhibition of PPAR gamma and Akt

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Abstract

We have previously reported that astaxanthin (AX), a dietary carotenoid, directly interacts with peroxisome proliferator-activated receptors PPARα and PPARγ, activating PPARα while inhibiting PPARγ, and thus reduces lipid accumulation in hepatocytes *in vitro*. To investigate the effects of AX *in vivo*, high-fat diet (HFD)-fed C57BL/6J mice were orally administered AX (6 or 30 mg/kg body weight) or vehicle for 8 weeks. AX significantly reduced the levels of triglyceride both in plasma and in liver compared with the control HFD mice. AX significantly improved liver histology and thus reduced both steatosis and inflammation scores of livers with hematoxylin and eosin staining. The number of inflammatory macrophages and Kupffer cells were reduced in livers by AX administration assessed with F4/80 staining. Hepatic PPARα-responsive genes involved in fatty acid uptake and β-oxidation were upregulated, whereas inflammatory genes were downregulated by AX administration. *In vitro* radiolabeled assays revealed that hepatic fatty acid oxidation was induced by AX administration, whereas fatty acid synthesis was not changed in hepatocytes. In mechanism studies, AX inhibited Akt activity and thus decreased SREBP1 phosphorylation and induced Insig-2a expression, both of which delayed nuclear translocation of SREBP1 and subsequent hepatic lipogenesis. Additionally, inhibition of the Akt-mTORC1 signaling axis by AX stimulated hepatic autophagy that could promote degradation of lipid droplets. These suggest that AX lowers hepatic lipid accumulation in HFD-fed mice via multiple mechanisms. In addition to the previously reported differential regulation of PPARα and PPARγ, inhibition of Akt activity and activation of hepatic autophagy reduced hepatic steatosis in mouse livers.

Keywords: Astaxanthin; PPAR; Akt; SREBP1; Autophagy

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a consequence of metabolic syndrome in the liver and is characterized histologically by hepatic steatosis that is not induced by alcohol consumption or other factors, such as viral infection, congenital and autoimmune liver diseases [1]. NAFLD is frequently associated with the chronic diseases obesity and diabetes, and the incidence of NAFLD has increased over the last decade. The initial presentation of NAFLD is simple steatosis, which then develops to steatohepatitis (NASH), characterized by hepatocyte injury and inflammatory infiltrates [2], and progresses to fibrosis and possibly cirrhosis, which is irreversible and even fatal [3]. Thus, control of fatty liver disease progression is an important way to prevent or treat NAFLD. The development of hepatic steatosis is considered to be the result of lipid accumulation in the liver, which is usually induced by an imbalance between lipid availability for hepatic lipid uptake (or *de novo* lipogenesis) and lipid disposal via fatty acid oxidation or triglyceride-rich lipoprotein secretion [1]. A hypothesis of NASH development suggests a "two-hit" model, in which the "1st hit" leads to steatosis and the "2nd hit" includes all factors promoting inflammation in the liver, which is responsible for the development of NASH [4]. The development of NASH from simple hepatic steatosis is highly associated with proinflammatory cytokines in the liver such as tumor necrosis factor-alpha (TNF-α) [5]. Thus, the regulation of the proteins that mediate hepatic lipid metabolism and inflammation may be a pharmaceutical target for prevention and treatment of NAFLD.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that belong to the steroid/thyroid hormone receptor superfamily and are activated by ligands to induce the transcription of target genes that regulate the metabolism of lipids, carbohydrates

Abbreviations: ACC1, acetyl-CoA carboxylase 1; ACOX1, acyl-CoA oxidase 1; Akt, protein kinase B; AX, astaxanthin; CPT1, carnitine palmitoyltransferase 1; FAS, fatty acid synthase; FPLC, fast protein liquid chromatography; GSK-3, glycogen synthase kinase-3; HFD, high-fat diet; IL-6, interleukin 6; Insig-2a, insulin-induced gene-2a; LPL, lipoprotein lipase; LXRα, liver X receptor alpha; mTOR, mammalian target of rapamycin; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; SCAP, SREBP-cleavage-activating protein; SREBP1c, sterol regulatory element binding protein 1c; S6K1, ribosomal protein S6 kinase 1; TNF-α, tumor necrosis factor-alpha; TZD, thiazolidinedione; UCP2, uncoupling protein 2.

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or proteins [6]. Of three PPAR isoforms, alpha (α), gamma (γ) and delta/beta (δ/β), PPARα is essential in the regulation of genes encoding fatty acid transport, metabolism and mitochondrial and peroxisomal fatty acid oxidation activity in the liver. Administration of PPARα agonists, such as fibrates widely used to treat hyperlipidemia, has been reported to ameliorate hepatic steatosis via improvement in mitochondrial fatty acid oxidation in mice [7]. On the other hand, PPARα-null (*Pparα*$^{-/-}$) mice showed a lower capacity of mitochondrial fatty acid oxidation compared to wild-type (*Pparα*$^{+/+}$) mice [8] and were susceptible to hepatic steatosis under high-fat diet (HFD) administration [9]. In addition, activation of PPARα exhibits antiinflammatory effects through a direct interaction of PPARα with nuclear factor kappa B (NF-κB) that inhibits NF-κB signaling and reduces NF-κB levels [10]. Thus, activation of PPARα is a pharmacological target for treatment of NAFLD. PPARγ is involved in regulation of glucose homeostasis, improves insulin sensitivity and is used as a major target for the thiazolidinediones (TZDs) in diabetes treatment [11]. Thus, activation of PPARγ by administration of TZDs improves hepatic steatosis in NAFLD, primarily via improved insulin sensitivity in adipose tissues [12]. PPARγ also regulates fatty acid storage via activation of genes that stimulate lipid uptake and adipogenesis [13]. Thus, overexpression of PPARγ induces lipid accumulation in adipose tissue and the liver. PPARγ is upregulated in steatotic livers compared with healthy livers [14], and overexpression of PPARγ activates *de novo* lipogenesis and increases triglyceride levels in the liver [15]. Treatment with TZDs induces increased lipid stores in hepatocytes and in rodent models [14,16]. The role of PPARγ in the liver was investigated in liver-specific PPARγ-knockout mice in which deletion of PPARγ in hepatocytes was protective against HFD-induced hepatic steatosis and downregulated genes involved in lipogenesis and hepatic lipid transport [17]. Thus, simultaneous activation of PPARα and inhibition of PPARγ may be a target for prevention and treatment of NAFLD.

In hepatic steatosis, activation of sterol regulatory element binding protein 1c (SREBP1c), a major lipogenic transcription factor, is critical. Its posttranslational regulation is well characterized. SREBP1 processing is initiated by chaperoning of the precursor SREBP1 protein (pSREBP) from the endoplasmic reticulum (ER) to the Golgi by the SREBP-cleavage-activating protein (SCAP). Then, two proteases, S1P and S2P, cleave pSREBP1 to release N-terminal SREBP1 to the nucleus (nSREBP) [18]. Insulin-induced gene-2a (Insig-2a, a liver-specific isoform of Insig) holds pSREBP in the ER to delay nuclear translocation of SREBP1 by complexing with SREBP1-SCAP when there are high levels of cellular sterol. Recently, the protein kinase B (Akt) was suggested to regulate SREBP1 nuclear translocation at multiple levels. First, Akt downregulates Insig-2a expression and thus induces ER-to-Golgi processing of SREBP1 [19]. Second, Akt promotes ER-to-Golgi transport of SREBP1 by directly phosphorylating SREBPs and facilitating the association of SREBP1 with coat protein complex II vesicles [20]. Third, Akt phosphorylates and inhibits activity of glycogen synthase kinase-3 (GSK-3), which regulates ubiquitin-dependent degradation of nSREBP [21]. Thus, Akt is a positive regulator for nuclear translocation of SREBP1 and thus induces hepatic lipogenesis.

The hepatic autophagy pathway is another mechanism involved in lipid metabolism. Hepatic autophagy is regulated by PPARs in multiple levels. The AMP-dependent kinase and PPARγ coactivator 1-alpha (PGC1-α), a PPARα-responsive gene, are suggested to activate autophagy pathway [22], whereas PPARγ blocks autophagy pathways [23]. Thus, activating PPARα and inhibiting PPARγ simultaneously may induce autophagy for clearance of undesirable lipid droplets via formation of autolysosomes and regulate proteins involved in lipid metabolism [24,25]. The hepatic autophagy pathway is suppressed by the Akt-mammalian target of rapamycin (mTOR) axis; thus, inhibition of mTOR activation may induce autophagy as well [26]. Therefore, regulation of PPAR subtypes and mTOR reduces lipid accumulation in the liver via induction of the hepatic autophagy pathway.

Our previous research suggested that astaxanthin (AX), a natural xanthophyll carotenoid abundant in marine organisms such as microalgae and salmon [27], is a PPARα agonist and a PPARγ antagonist and ameliorates lipid accumulation in cultured hepatocytes by regulating genes involved in lipid metabolism in HepG2 cells [28]. AX has antioxidant and antiinflammatory activity as well as antisteatotic and antioxidant properties in the liver that prevent the development of NAFLD [29]. The administration of AX to rats treated with carbon tetrachloride (CCl_4, a chemical inducer of NASH) led to inhibition of lipid peroxidation, increased the levels of glutathione and activated superoxide dismutase [30]. AX reduces HFD-induced weight gain, adipose and liver weights and hepatic triglyceride levels by inducing energy expenditure by increasing the utilization of fatty acids in ddY mice [31]. In high fat/high fructose diet-fed mice, AX administration reduces lipid accumulation and peroxides, thus improving hepatic antioxidant status [29]. AX also prevents the progression of NASH and may have hepatoprotective effects against existing hepatic injury [32]. Therefore, AX could be a natural compound used for the prevention and treatment of NAFLD and NASH. In this study, we investigated whether AX could ameliorate hepatic steatosis *in vivo* and the molecular mechanisms of actions of AX on the hepatic lipid metabolism. For the experiments, C57BL/6J mice were fed HFD for 8 weeks to induce hepatic steatosis then AX was orally administered for additional 8 weeks under HFD feeding.

2. Materials and methods

2.1. Reagents and materials

The medium and cell culture supplies were purchased from Hyclone (Logan, UT, USA). The AX (596.84 g/mol) was purchased from Hangzhou Toyond Biotech (Hangzhou, China). The RNAiso Plus used to extract total RNA and SYBR Premix Ex Taq used for real-time PCR were purchased from Takara (Otsu, Japan). The monoclonal antibodies anti-PPARα, anti-PPARγ, anti-SREBP1, anti-Insig-2a, anti-β-actin, F4/80 antibody and antimouse and antirabbit immunoglobulin G were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-phospho-Akt and anti-LC3 antibodies were obtained from Novus (Littleton, CO, USA). Antibodies to phospho-SREBP1c, ribosomal protein S6 kinase 1 (S6K1) and phospho-S6K1 were obtained from Cell Signaling Technology (Danvers, MA, USA). The Autophagy Protein Detection Set (containing the anti-lysosomal-associated membrane protein LAMP1, anti-LAMP2, the antiautophagy-related protein APG7 and anti-Beclin) was purchased from ProSci (Poway, CA, USA).

2.2. Cell culture and maintenance

HepG2 and HEK293 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in a Dulbecco's modified Eagle's medium (Hyclone) medium with 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 of 5% CO_2.

2.3. Mouse feeding and care

Eight-week-old C57BL/6J male mice (18–20 g; Samtako Co., Gyeonggi-Do, Korea) were randomly assigned to four groups (n=10 for each group): standard diet (SD), HFD (45% of total calories from fat; Central Lab. Animal Inc., Seoul, Korea), HFD with a low dose of AX (AX6; 6 mg/kg body weight of AX) and HFD with a high dose of AX (AX30; 30 mg/kg body weight of AX). Mice were maintained on a 12-h light/dark cycle with a controlled temperature of 21–25°C and humidity of 50–60%, according to a protocol approved by the Animal Experiment Committee of Korea University (Protocol No. KUIACUC-2013-139). Mice were fed a commercial chow diet or HFD for 8 weeks to induce hepatic steatosis in HFD groups. The mice were then given olive oil (control) or AX dissolved in olive oil (3 ml/kg body weight) by oral gavage, at evening, for another 8 weeks. At the end of the experimental period, mice were killed after 12 h of fasting. Plasma and tissue samples were collected according to a protocol approved by the Animal Experiment Committee of Korea University (Protocol No. KUIACUC-2013-139). All samples were stored at −80°C for further use.

2.4. Quantification of lipids and hormones

Hepatic lipids and proteins were extracted and measured by an automated clinical chemistry analyzer (Cobas111; Roche, Basel, Switzerland) as described previously [33]. The plasma total cholesterol, high-density-lipoprotein (HDL) cholesterol, low-density-lipoprotein (LDL) cholesterol, triglyceride and glucose levels as well as hepatic