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 PPARs and PPARγ, activating PPARs 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 PPARs-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 inhibition of PPARs 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 of 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; T2D, thiazolidinedione; UCP2, uncoupling protein 2.

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or proteins [6]. Of three PPAR isofoms, 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<sup>−/−</sup>) mice showed a lower capacity of mitochondrial fatty acid oxidation compared to wild-type (Pparα<sup>+/+</sup>) 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 increases induced lipids 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, SIP and S2P, cleave pSREBP1 to release N-terminal SREBP1 to the nucleus (nSREBP1) [18]. Insulin-induced gene-2a (Insig-2a, a liver-specific isoform of Insig) holds pSREBP1 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 nSREBP1 [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 antistatotic and antioxidant properties in the liver that prevent the development of NAFLD [29]. The administration of AX to rats treated with carbon tetrachloride (CCl<sub>4</sub>, 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-PPARγ, anti-PPARα, anti-SREBP1, anti-Insig-2a, anti-α-actin, F4/80 antibody and antimonouse antirabbit immunoglobulin G were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, 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% CO2.

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 (AXG; 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. KUJACUC-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. KUJACUC-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
between the groups.

The data are presented as the mean±S.E.M. Significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the groups.

cholesterol and triglyceride concentrations were measured by Cobas111 analyzer according to the manufacturer’s instructions. Plasma and hepatic TNF-α and interleukin 6 (IL-6) concentrations were quantified by ELISA (Thermo Scientific, Colorado, USA) according to the manufacturer’s instructions.

2.5. Histological analysis

The hematoxylin and eosin (H&E) staining of liver and epididymal adipose tissues was performed by the Histopathology Department of Anam Korea University Hospital (Seoul, Korea). Tissues were fixed in 4% paraformaldehyde after removal and stained with H&E. The size of adipocytes was measured by an upright microscope (Axio Imager M1; Carl Zeiss, Oberkochen, Germany). Immunohistochemistry was performed by incubating the liver sections in F4/80 antibody and using the Immunocruz rat ABC Staining System (Santa Cruz, CA, USA) according to the manufacturer’s instructions. Briefly, the liver sections were incubated in deionized H2O to quench endogenous peroxidase activity and then washed by PBS twice and incubated in 1% blocking serum for 30 min at room temperature and then washed by PBS three times. Sections were then incubated in biotinylated secondary antibody at room temperature for 30 min and washed three times by PBS. Next, samples were incubated in avidin and biotinylated HRP (1:1) mixed solution for 30 min at room temperature and then washed by PBS three times. The inflammatory cells were stained by incubating the liver sections in peroxidase substrate for 10 min and washed by deionized H2O. The hematoxylin was used to stain the nuclei.

2.6. NAFLD scoring system

We used an NAFLD scoring system for rodent models established by the NASH Clinical Research Network [34,35] to assess the histological score of NAFLD of livers. In this NAFLD scoring system, the two key features of NAFLD, steatosis and inflammation, were determined. First, steatosis was measured by the presence of microvesicular steatosis, macrovesicular steatosis and hepatocellular hypertrophy of hepatocytes. Microvesicular steatosis means that hepatocytes with small lipid droplets with the nucleus are in the center of cells, macrovesicular steatosis indicates that hepatocytes with large lipid droplets are displaced the nucleus to the side and hypertrophy means that cells have much larger size than the steatotic hepatocytes (more than 1.5 times) but have the same cytoplasmic characteristics. The severity of steatosis was graded based on the percentage of the total area affected as follows: 0 (0%–5%), 1 (5–33%), 2 (34–66%) and 3 (>66%). Thus, the score of hepatic steatosis was the sum of the scores of microvesicular steatosis, macrovesicular steatosis and hypertrophy, ranged from 0 to 9. Then the inflammation was scored by counting the number of inflammatory foci (a cluster of more than five inflammatory cells) per field (area of 3.1 mm²). The score of inflammation was graded as follows: 0 (<0.5 foci), 1 (0.5–1.0 foci), 2 (1.0–2.0 foci) and 3 (>2.0 foci), by calculating the average of five different fields.

Table 1

<table>
<thead>
<tr>
<th>Plasma cholesterol, triglyceride and glucose concentrations of mice fed by AX.</th>
<th>Plasma (mg/dl)</th>
<th>SD</th>
<th>HFD</th>
<th>HFD+AX6</th>
<th>HFD+AX30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>116.7±16.0</td>
<td>225.3±22.0</td>
<td>208.1±08.1</td>
<td>216.0±16.0</td>
<td>216.0±16.0</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>73.7±3.7</td>
<td>97.7±7.7</td>
<td>69.1±9.1</td>
<td>68.0±8.9</td>
<td>68.0±8.9</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>40.8±0.3</td>
<td>132.7±32.3</td>
<td>131.7±31.7</td>
<td>150.3±50.3</td>
<td>150.3±50.3</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>224.3±24.3</td>
<td>271.8±71.8</td>
<td>281.1±81.1</td>
<td>265.2±65.2</td>
<td>265.2±65.2</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>156.4±56.0</td>
<td>244.1±44.0</td>
<td>247.3±47.3</td>
<td>245.2±45.2</td>
<td>245.2±45.2</td>
</tr>
</tbody>
</table>

The data are presented as the mean±S.E.M., and significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the groups.
2.10. Akt kinase assay

HepG2 cells were cultured in 12-well plates at density of 5 × 10⁵ per well for 24 h. Palmitic acid and oleic acid (400 μM for each) were added to the cells with 0.5% BSA (Bovogen Biologicals, Melbourne, Australia) for 24 h to induce cellular lipid accumulation. Cells were treated with 6 μM or 30 μM of AX for another 24 h. The oxidation and synthesis of fatty acids was investigated by measuring the [1-¹⁴C]plamitate and [1-¹⁴C]acetate (45–60 nCi/mmol; Perkin Elmer) levels, respectively, as described previously [37]. The radioactivity of [1-¹⁴C]plamitate-labeled CO₂ was measured to represent the fatty acid oxidation of HepG2 cells, and the radioactivity of [1-¹⁴C]acetate-labeled lipid was assessed to represent the fatty acid synthesis of HepG2 cells. The protein concentration was assessed by the Bio-Rad reagent (Bio-Rad) and used to normalize the radioactivity data.

2.9. Quantitative PCR (qPCR) analysis

Extraction of total RNA and synthesis of cDNA was performed as described previously [38]. Livers were homogenized in RNAiso Plus reagent and the total RNA was extracted according to the manufacturer’s instructions. The synthesis of cDNA was performed by mixing 2 μg of total RNA with the M-MLV reverse transcriptase, oligo-dT and dNTPs (MBIotech, Korea). Real-time qPCR was performed using the Bio-Rad IQ5 Cycler System, using the glyceraldehyde 3-phosphate dehydrogenase gene for normalization. Primers were shown in Supplementary Table 3.

2.10. Akt kinase assay

The hepatic Akt activity was assessed by an Akt kinase assay (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer’s instructions. In brief, mouse livers (10 mg for each liver) were incubated in lysis buffer [20 mM Tris (pH 7.5), 150 mM EDTA, 1 mM EGTA, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄ and 1 μg/ml leupeptin] for 5 min and then sonicated on ice. After centrifugation at 13,000g for 10 min, immunoprecipitation was performed. The supernatant was incubated with beads immobilized with Akt primary antibody at 4°C overnight with gentle rocking. Then the mixture was centrifuged at 14,000g for 30 s to collect pellet, which then was washed by lysis buffer and kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄ and 10 mM MgCl₂] twice, respectively. Pellets were suspended in 50 μl kinase buffer supplemented with 1 μl of 10 mM ATP and 1 μl of GSK-3 fusion protein and were incubated at 30°C for 30 min. The reaction was terminated with 25 μl of 3× SDS sample buffer then centrifuged at 14,000g for 30 s to get supernatants. The supernatant was heated and loaded on SDS-PAGE, and subsequent immunoblotting was performed. The phosphor-GSK-3 (Ser21/9) antibody was used as primary antibody and the HRP-conjugated secondary antibody and HRP-conjugated antibiotin antibody were used as the secondary antibody. The immunoblotting image was obtained by using a ChemiDoc XR+ imaging system (Bio-Rad).

2.11. Immunoblotting analysis

Protein was extracted from the liver by homogenizing the liver in RIPA buffer. Samples were centrifuged at 14,000 rpm for 10 min at 4°C to collect the supernatant. Protein concentration was determined using a Bio-Rad reagent (Bio-Rad). Protein was denatured by heating and run on SDS-PAGE as described previously [33]. The protein on SDS-PAGE was transferred to the membrane, which was incubated in primary and secondary antibodies to obtain the immunoblotting image using a ChemiDoc XR+ imaging system (Bio-Rad).

2.12. Fluorescent microscopic analysis

HEK293 cells, cultured in 6-well plates at 1.5 × 10⁵ cells per milliliter, were transfected with tandem fluorescent mRFP-GFP-LC3 plasmids (Addgene, Cambridge, MA, USA) using FuGENE HD (Promega, Madison, WI, USA) according to the manufacturer’s instructions. After selection with G418 antibiotics (Sigma, St. Louis, MO, USA) for several days, the selected cells were fixed with 4% paraformaldehyde and stained with DAPI (Invitrogen) for nuclei and mounted with antifade reagent. Image was taken on a Zeiss LSM 5 Exciter confocal microscope (100×/1.30 oil DIC) and analyzed by Zeiss LSM510 v.3.2 software (Carl Zeiss, Jena, Germany). LC3 in
Autophagosome emits only red fluorescence and visualized as red puncta. LC3 expression was calculated by counting the number of positive red puncta in three random areas for each group.

2.13. Statistical analysis

All data are shown as the mean ± S.E.M, and one-way ANOVA was used to calculate the significant differences between the groups. A value of P < 0.05 was considered significant.

3. Results

3.1. AX decreased plasma and hepatic lipid concentrations and reduced epididymal fat and adipocyte size without changing the body weight of mice

AX is a natural compound with hepatoprotective effects [29]. Here, we investigated the effect of AX on HFD-fed C57BL/6J and found that both 6 mg/kg and 30 mg/kg of AX significantly reduced triglyceride concentration in both plasma (Table 1) and livers (Fig. 1A). Histological analysis of the liver showed a similar trend (Fig. 1B and C). Plasma aspartate transaminase levels were similar among four groups while alanine transaminase levels were higher in HFD groups (HFD, HFD+AX6 and HFD+AX30) compared with those of normal-chow-fed controls. HFD-fed mice in this study did not develop a severe liver injury; thus, moderately elevated levels of alanine transaminase may be a result of moderate lipid accumulation in mice fed with HFD or HFD and AX (Table 1). We performed histological analysis based on NAFLD scoring system [34,35]. The histological analysis of H&E-stained livers showed that AX administration reduced NAFLD steatosis scores. Steatosis score of HFD-fed liver was 2 but was lowered to 1 in HFD-AX livers. Since steatosis score 2 indicates the presence of both macrovesicular steatosis and microvesicular steatosis while score 1 means the existence of microvesicular steatosis but not macrovesicular steatosis; these steatosis score changes suggest the amelioration of hepatic lipid accumulation in HFD-fed mice (Fig. 1B).

Furthermore, the inflammatory foci, inflammatory macrophages and Kupffer cells were analyzed in livers with both H&E and F4/80 staining (Fig. 1B and C). In the H&E-stained livers, the NAFLD inflammation score of HFD-fed control liver was 1 but was decreased to 0 in HFD-AX-fed livers. Inflammation score of 1 indicates the appearance of 0.5–1.0 foci whereas the inflammation score of 0 means the observance of <0.5 foci [34,35] (Fig. 1B). In addition, the livers with F4/80 staining showed significantly reduced inflammatory macrophages and Kupffer cells in HFD-AX6-fed livers, and these inflammatory cells were not observed in HFD-AX30-fed livers, compared with controls (Fig. 1C). These results collectively suggest that AX significantly reduced hepatic inflammation.

Body weights, food intake, hepatic cholesterol and liver weight were not changed (Supplementary Fig. 1 and Supplementary Tables 1 and 2). However, AX administration significantly reduced the epididymal fat weight and adipocyte size compared with controls (Fig. 1D). The concentration of HDL cholesterol was significantly increased by 11% in AX30 mice (Fig. 2), whereas LDL and total cholesterol levels were unaltered in the AX groups compared with the HFD group. The results suggested that AX administration decreased plasma, liver and adipose fat accumulation with ameliorating hepatic steatosis.

3.2. AX-induced hepatic fatty acid β-oxidation did not alter cellular fatty acid synthesis

Hepatic lipid homeostasis is maintained by a balance of fatty acid oxidation and synthesis. HepG2 cells stimulated with AX (6 μM and 30 μM) were investigated for fatty acid oxidation and synthesis using radiolabeled [1-14C]palmitate and [1-14C]acetate, respectively.

AX stimulation significantly increased fatty acid oxidation by 17% at 30 μM in HepG2 cells, whereas fatty acid synthesis was unaffected compared with controls without AX treatment (Fig. 3A), which suggested that the reduction of hepatic and plasma triglyceride concentrations in mice given by AX was achieved by increased hepatic β-oxidation.

3.3. AX upregulated PPARα and downregulated PPARγ and gene and protein expression involved in hepatic lipid metabolism

The nuclear receptors PPARα and PPARγ are key regulators of lipid metabolism in liver. We have previously reported that AX directly interacts with PPARs and functions as an agonist of PPARα and antagonist of PPARγ and thus reduces lipid accumulation in hepatocytes in vitro [28]. In this study of mouse livers, qPCR and immunoblotting analyses showed that both AX6 and AX30 feeding significantly increased PPARα gene and protein expression compared with the HFD group, and AX30 feeding reduced PPARγ gene and protein expression compared with the HFD group (Fig. 3B and C). Next, we investigated the expression of genes involved in hepatic lipid metabolism (Fig. 3D). The genes involved in fatty acid uptake are regulated by both PPARα and PPARγ. AX administration significantly increased the mRNA expression of caveolin 1, a membrane protein abundant in hepatocytes that creates highly ordered domains at the cell surface for fatty acid uptake [39]. However, fatty acid transmembrane translocases [1], such as CD36, the fatty acid transport protein FATP5 and the fatty acid binding protein FABP1, were not altered by AX feeding, suggesting that the hypotriglyceridemic effect of AX may be partially achieved by induction of fatty acid uptake in the livers of mice.

PPARα regulates the expressions of key proteins involved in mitochondrial and peroxisomal β-oxidation, as well as genes involved in fatty acid utilization and catabolism. The mitochondrial uptake of long-chain fatty acid is mediated by carnitine palmityltransferase 1 (CPT1), the induction of which leads to mitochondrial β-oxidation [6]. In addition, acyl-CoA oxidase 1 (ACOX1), which catalyzes fatty acyl-CoAs to their corresponding trans-2-enoyl-CoAs in the peroxisomal β-oxidation, is a key enzyme that is regulated in a PPAR-dependent manner in the liver [40]. The mRNA expressions of CPT1 and ACOX1 were significantly upregulated by AX administration compared with controls (Fig. 3D). PPARα regulates lipoprotein lipase (LPL), a triglyceride hydrolase that reduces triglyceride levels and mitochondrial thermogenesis by uncoupling protein 2 (UCP2), a regulator of mitochondria-derived reactive oxygen species in hepatic lipid metabolism. AX administration also upregulated the expression of LPL and UCP2, which contribute to the reduction of adipose tissue and hepatic lipid accumulation. Liver X receptor alpha (LXRXα), a target gene of PPARα that is involved in lipogenesis, was induced by AX30 (47%), whereas hepatic lipoprotein transcription factor and genes, including SREBP1c, fatty acid synthase (FAS) and acetyl-CoA carboxylase 1 (ACC1), were unchanged in AX livers. Other genes regulating lipogenesis, such as the carbohydrate-responsive element binding protein ChREBP, were regulated by PPARα. The diacylglycerol acyltransferase 1 was regulated by PPARγ and was unaltered by AX treatment. The expression of PPARα and PPARγ and their responsive genes indicated that AX produces hypotriglyceridemic effects via activation of PPARα and inhibition of PPARγ, as well as by regulation of responsive genes in hepatic lipid metabolism.

3.4. AX reduced hepatic steatosis by inhibiting the Akt-mTOR axis and activating the autophagy pathway

Hepatic steatosis is regulated by fatty acid uptake, oxidation and synthesis. SREBP1c is a critical transcription factor in hepatic lipogenesis. The posttranslational modification of SREBP1 is initiated by
translocation of the precursor SREBP1 protein, chaperoned by the SCAP from the ER to the Golgi. This translocation is stimulated by phosphorylation of SCAP. The precursor SREBP1 (pSREBP1) protein is cleaved by two distinct proteases, S1P and S2P, to release the mature form of SREBP1. Insig-2a enhances the amount of SREBP1 on the ER membrane, inhibiting the processing of SREBP1 [41,42]. Akt phosphorylates GSK-3 that accelerates SREBP1 degradation by proteasome-dependent manner [21]. Akt downregulates Insig-2a and thus promotes ER-to-Golgi transport of SREBP1. Akt directly phosphorylates SREBPs, which facilitates the incorporation of pSREBP1-SCAP complex to coat protein complex II vesicles to mediate ER-to-Golgi transport. First, we assessed the Akt activity with AX treatment. The Akt from mouse livers was incubated with GSK-3 fusion protein to phosphorylate GSK-3, and then GSK-3 phosphorylation was assessed using phospho-GSK-3 (Ser21/9) antibody. The results showed that AX30 significantly reduced Akt activity and GSK-3 (Ser21/9) phosphorylation of mouse livers. (Fig. 4A). Second, in the immunoblotting, we found that both AX6 and AX30 feeding significantly reduced the phosphorylation of SREBP1 was significantly reduced by AX stimulation in the mouse liver (Fig. 5A). Simultaneously, AX administration induced the expression of major cellular markers involved in the autophagy pathway. AX feeding increased the protein expression of the autophagy marker LC3II by 187% and increased the ratio of LC3II/LC3I significantly. Other key proteins involved in the autophagy pathway such as LAMP1, LAMP2, APG7 and beclin1 were increased compared with HFD livers (Fig. 5A). In mRFP-GFP-LC3 transfected HEK293 cells such as LAMP1, LAMP2, APG7 and beclin1 were increased compared with HFD livers (Fig. 5A). In mRFP-GFP-LC3 transfected HEK293 cells such as LAMP1, LAMP2, APG7 and beclin1 were increased compared with HFD livers (Fig. 5A). In mRFP-GFP-LC3 transfected HEK293 cells such as LAMP1, LAMP2, APG7 and beclin1 were increased compared with HFD livers (Fig. 5A). In mRFP-GFP-LC3 transfected HEK293 cells such as LAMP1, LAMP2, APG7 and beclin1 were increased compared with HFD livers (Fig. 5A).

Activation of hepatic autophagy has been reported to regulate lipid metabolism via the breakdown of lipid droplets in the liver, thus reducing lipids storage. Induction of autophagy is regulated by PPARα and PPARγ. The results demonstrated that the expression of genes involved in lipid metabolism. The data are presented as the mean±S.E.M. Significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the groups.

3.5. AX reduced inflammation in the plasma and liver

Inflammation is a key biological component associated with the development of NAFLD to NASH. We quantified key proinflammatory markers and found that TNF-α levels were significantly reduced low and high levels of AX feeding in both plasma (27% for AX30 mice) and livers (63% for AX30 mice) compared with HFD control mice. The level of IL-6 in the plasma and liver was reduced by high dose of AX by 27% and 34%, respectively, compared with HFD-fed mice (Fig. 6A and B).
The expression levels of TNF-α and IL-6 were also reduced significantly by low and high levels of AX administration (Fig. 6C). Thus, AX ameliorated hepatic inflammation compared with HFD control mice, which indicated that AX blocks the development of NASH via inhibiting proinflammatory markers in the mouse liver.

4. Discussion

We previously demonstrated that AX is an agonist for PPARα and an antagonist for PPARγ and that AX reduces intracellular lipid levels by regulating genes involved in fatty acid and cholesterol biosynthesis in cultured hepatocytes in vitro [28]. Here, we further demonstrated effect of AX in vivo that AX administration in mice activates PPARα, inducing its mRNA and protein expression while inhibiting PPARγ and its expression in the mouse liver. AX administration ameliorates HFD-induced hepatic lipid accumulation and epididymal fat weight as well as adipocyte size in mice, and it also reduces plasma and hepatic triglyceride levels. These metabolic outcomes were due to the combined regulatory roles of AX on both PPARα and PPARγ. This can be concluded given that the activation of PPARα improves fatty acid transport, metabolism and oxidation in the liver [43], while inhibition of PPARγ reduces lipogenesis in adipose tissue and the liver [15]. The balance between fatty acid oxidation and synthesis is a key regulator in the liver and mediates the development of hepatic steatosis. We showed that AX administration increases the expression of genes involved in fatty acid oxidation, whereas the synthesis of fatty acids in hepatocytes was not changed. These data confirm the agonistic effects of AX on PPARα and the antagonistic effects of AX on PPARγ as well.

Excessive lipogenesis is a major factor that induces hepatic steatosis in liver. LXRα is a major hepatic regulator of cholesterol absorption, transport, efflux and excretion, and it mediates cholesterol homeostasis of the body. LXRα is also a PPARα target gene [44]. Activation of LXRα increases HDL levels in rodent models by inducing responsive genes such as ATP-binding cassette transporter ABCA1 (ABCA1) and the ATP-binding cassette subfamily ABCG1, which are involved in cholesterol transport to regulate cellular lipid homeostasis [45]. AX administration increases hepatic LXRα expression, which may contribute to an increase of plasma HDL cholesterol levels in the AX groups. Genes involved in hepatic lipogenesis, such as SREBP1c and its targets FAS and ACC1, were slightly increased by AX administration compared with HFD livers but were expressed at similar levels as in ND-fed livers. However, pSREBP1 protein expression was not induced, and AX stimulation reduced nSREBP1 compared to HFD livers. These suggest that posttranslational processing of SREBP1 was blocked by AX feeding. Akt regulates the processing of SREBP1 by enhancing SREBP1c phosphorylation, downregulating the expression of the liver-specific gene Insig-2a and inhibiting GSK-3. Our results showed that AX stimulation inhibited Akt phosphorylation, induced Insig-2a expression and reduced SREBP1 and GSK-3 phosphorylation, which...
collectively contribute to reduce translocation and processing of SREBP1 and blocking hepatic lipogenesis in a SREBP1-dependent manner (Fig. 7).

Akt activation is also regulated by PPARs. Activation of PPARα inhibits Akt by inhibiting its phosphorylation [46]. However, the effect of PPARγ on the Akt pathway is still controversial. Activation of PPARγ by agonist such as rosiglitazone induces phosphorylation of Akt, thus activating Akt [47]. In contrast, another agonist, troglitazone, inhibits the phosphorylation of Akt [46]. Our previous research indicated that AX has a much stronger agonistic effect on PPARγ than rosiglitazone [28]. Thus, the reduction of Akt activation may be achieved by a combined effect of PPARα activation and PPARγ inhibition in AX-fed mouse livers.

Induction of hepatic autophagy pathway is beneficial to lipid metabolism by breaking down stored lipid droplets [25]. Induction of the hepatic autophagy pathway is regulated by inhibition of the Akt-mTOR pathway [26]. Akt activates mTORC1, resulting in phosphorylation of S6K1. Our results confirmed that AX induced the hepatic autophagy pathway via inhibition of the Akt-mTOR pathway in mouse livers. In addition, the autophagy pathway is regulated in a PPAR-dependent manner. PPARα may induce autophagy pathway via AMP-activated protein kinase or PGC1-α. However, PPARγ agonists inhibit autophagy [23], and PPARγ knockdown induces the autophagy marker LC3II [48]. Thus, AX, an agonist of PPARα and an antagonist of PPARγ, induces markers of the autophagy pathway in the livers of mice, inducing the clearance of lipids in the liver and the whole body. Therefore, induction of hepatic autophagy may be due to the combined effects of PPARα activation and PPARγ and Akt inhibition (Fig. 7).

NASH involves hepatic lipid accumulation combined with hepatic inflammation. Our results showed that two proinflammatory cytokines, TNF-α and IL-6, were reduced in plasma and liver by AX administration. Hepatic mRNA expression was also reduced, suggesting an antiinflammatory effect of AX in HFD-fed mice. Activation of PPARα could inhibit both TNF-α and IL-6; thus, the antiinflammatory effects of AX may be achieved by activation of PPARα.

In conclusion, AX administration reduces hepatic lipid plasma triglyceride concentration and reduces epididymal fat and adipocyte size in HFD-fed mice by activating PPARα and inhibiting PPARγ. The net effect of AX is to induce fatty acid oxidation and hepatic autophagy by direct regulation of PPARα and PPARγ. AX also has an inhibitory effect on the Akt-mTOR pathway and thus hepatic lipid metabolism. Furthermore, AX administration reduces the proinflammatory cytokines TNF-α and IL-6 in the liver and circulatory system via activation of PPARα as well. Therefore, AX administration improves hepatic steatosis and inflammation by primarily regulation of PPARα and PPARγ and potential inhibitory effect on Akt and may be beneficial for the prevention and treatment of NAFLD.
Conflicts of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.09.015.

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