Astaxanthin inhibits inflammation and fibrosis in the liver and adipose tissue of mouse models of diet-induced obesity and nonalcoholic steatohepatitis

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Abbreviations: ACOX-1, acyl-coenzyme A oxidase 1; ALT, alanine aminotransferase; apoE, apolipoprotein E; Arg-1, arginase-1; ASTX, astaxanthin; Casp, Caspase; CVD, cardiovascular disease; CPT-1 α , carnitine palmitoyltransferase 1 α ; CD68, cluster of differentiation 68; CD206, cluster of differentiation 206; COL1A1, collagen type I, α 1; COL6A, collagen type VI, α ; DIO, diet-induced obesity; ECM, extracellular matrix, FAS, fatty acid synthase; H&E, hematoxylin and eosin; HIF1- α , hypoxia-inducible factor 1- α ; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; IL, interleukin; HSCs, hepatic stellate cells; LDLR, LDL receptor; LOXL2, lysyl oxidase-like 2; LPS, lipopolysaccharide; LUM, lumican; MMP-2, matrix metalloproteinase-2; MCP-1, monocyte chemoattractant protein 1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; PGC-1, PPAR γ

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3 co-activator 1; ROS, reactive oxygen species; SCD-1, stearyl CoA desaturase 1; SREBP, sterol
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6 regulatory element binding protein; TNC, tenascin-C; TGF β 1, transforming growth factor β 1;
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8 TLR4, toll-like receptor 4; TNF α , tumor necrosis factor α ; TC, total cholesterol; TG,
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10 triglyceride; UCP, uncoupling protein; VIM, vimentin.
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Abstract

The objective of this study was to determine if astaxanthin (ASTX), a xanthophyll carotenoid, can prevent obesity-associated metabolic abnormalities, inflammation and fibrosis in diet-induced obesity (DIO) and nonalcoholic steatohepatitis (NASH) mouse models. Male C57BL/6J mice were fed a low fat (6% fat, w/w), a high fat/high sucrose control (HF/HS; 35% fat, 35% sucrose, w/w), or a HF/HS containing ASTX (AHF/HS; 0.03% ASTX, w/w) for 30 wk. To induce NASH, another set of mice was fed a HF/HS diet containing 2% cholesterol (HF/HS/HC) or HF/HS/HC with 0.015% ASTX (AHF/HS/HC) for 18 wk. Compared to LF, HF/HS significantly increased plasma total cholesterol, triglyceride and glucose, which were lowered by ASTX. ASTX decreased hepatic mRNA levels of markers of macrophages and fibrosis in both models. The effect of ASTX was more prominent in NASH than DIO mice. In epididymal fat, ASTX also decreased macrophage infiltration and M1 macrophage marker expression, and inhibited hypoxia-inducible factor 1- α and its downstream fibrogenic genes in both mouse models. ASTX significantly decreased tumor necrosis factor α mRNA in the splenocytes from DIO mice upon lipopolysaccharides stimulation, compared with those from control mice fed a HF/HS diet. Additionally, ASTX significantly elevated the levels of genes that regulate fatty acid β -oxidation and mitochondrial biogenesis in the skeletal muscle, compared with control obese mice, whereas no differences were noted in adipose lipogenic genes. Our results indicate that ASTX inhibits inflammation and fibrosis in the liver and adipose tissue and enhances the skeletal muscle's capacity for mitochondrial fatty acid oxidation in obese mice.

Keyword: Astaxanthin; Obesity; Macrophage infiltration; Inflammation; Fibrosis; Macrophage phenotypes

1. Introduction

Obese individuals have a high risk of developing non-alcoholic fatty liver disease (NAFLD) [1]. When adipocytes become hypertrophied, macrophages infiltrate into the adipose tissue and produce pro-inflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin 6 (IL-6) [2-4]. The cytokines stimulate the release of free fatty acids from adipocytes, resulting in high influx of free fatty acids into the liver, leading to the development of liver steatosis [5]. Steatosis can progress to non-alcoholic steatohepatitis (NASH), which is characterized by inflammation and fibrosis [6-8]. Liver fibrosis is a scarring process that can progress to cirrhosis, the primary cause for hepatocellular carcinoma [9, 10]. Fibrosis in adipose tissue is also an important hallmark of dysfunctional adipose tissue, which is closely associated with adiposity in mice and humans [11-14]. While adipocyte expansion is necessary to accommodate fat storage, excessive and rigid extracellular matrix (ECM) surrounding adipocytes can cause local fibrosis and inflammation [15]. Although the etiology and metabolic consequences of adipose tissue fibrosis are not well understood, recent studies have shown that fibrosis may be a cause or a result of adipose tissue inflammation in obesity [16].

Based on chemokine receptor expression and specific surface molecules, tissue macrophages can be classified as classically-activated, pro-inflammatory M1 and alternatively-activated, anti-inflammatory M2 type [17, 18]. CD11c is a well-known marker of M1 macrophages [19], which produce pro-inflammatory cytokines, such as TNF α , IL-6, and monocyte chemoattractant protein-1 (MCP-1) [20-22]. M2 macrophages are the major resident macrophages in lean adipose tissue and are characterized by high expression of CD206, arginase-1 (Arg-1), macrophage galactose-type calcium-type lectin 1/CD301a, and IL-10, which are involved in the repair or remodeling of the tissue [19-22]. Recent studies have shown that M1

and M2 macrophages are involved in adipose insulin resistance [23-25]. Importantly, the increased number of M1 macrophages and high M1-to-M2 ratios are related to the development of insulin resistance in mice fed a high fat diet [26]. At present, however, very little information is available on dietary components that regulate the phenotypic switch of M1 to M2 macrophages.

Astaxanthin (ASTX) is a xanthophyll carotenoid abundant in marine animals such as salmon and shrimp. It contains hydroxyl and keto moieties on both ends of its structure with a potent antioxidant property [27-29]. ASTX has been shown to be protective against oxidative stress, inflammation, diabetes, cardiovascular disease (CVD) and NAFLD [30-35]. However, the mechanisms underlying such effects remains unclear. We previously reported that ASTX prevented dyslipidemia and improved antioxidant defense mechanisms in apolipoprotein E knockout (*apoE^{-/-}*) mice [36] and diet-induced obesity (DIO) mice [37]. Furthermore, we also found that ASTX prevented pro-fibrogenic gene expression in mouse primary hepatic stellate cells (HSCs) and LX-2 cells, a human hepatic stellate cell line [38]. In the present study, we sought to investigate if ASTX supplementation can prevent obesity-associated inflammation and fibrosis in mouse models of DIO and NASH.

2. Material and Methods

2. 1. Animal care and diet

Male C57BL/6J mice at 8 weeks age were purchased from Jackson Laboratory (BarHarbor, ME) and randomly assigned to a low fat control (LF; 6% fat by wt, n=7), a high fat/high sucrose control (HF/HS; 35% fat, 35% sucrose by wt, n=9) or a HF/HS diet containing 0.03% ASTX by wt (AHF/HS, n=9). AstaREAL[®] Oil 50F, an extract from a microalgae

Haematoccus pluvialis containing 5% of ASTX, was generously provided by Fuji Chemical Industry Co., LTD. (Toyama, Japan). The composition of the experimental diets is shown in **Table 1**. In another study, male C57BL/6J mice were randomly assigned to either a control high fat/high sucrose/high cholesterol diet (HF/HS/HC: 35%/34%/2.0% by wt, n=3) or the HF/HS/HC diet supplemented with 0.015% ASTX by wt (AHF/HS/HC, n=3) for 18 weeks to induce liver fibrosis. Sucrose (0.2%) was added in drinking water throughout the feeding period. Dietary cholesterol at 2% was added because the cholesterol level was shown to induce NASH in mice [42]. The composition of HF/HS/HC diet is similar to HF/HS diet, except the inclusion of cholesterol. Mice were housed in a polycarbonate cage under a 12 h light/dark cycle. Body weights and food consumption were recorded weekly, and blood samples were collected monthly from the lateral tail vein. At the end of the feeding period, mice were starved for 8 h and anaesthetized by ketamine/xylazine (120/6 mpk) (Henry Schein Animal Health, Dublin, OH). Blood was collected in a tube containing 3.5 mg EDTA (BD Vacutainer) and centrifuged at 1000 x g for 15 min at 4°C for plasma collection. Liver, epididymal adipose tissue (eAT), retroperitonea adipose tissue (rAT), gastrocnemius muscle, and spleen were harvested and snap frozen in liquid nitrogen or fixed in 10% formalin. Plasma and tissue samples were stored at -80°C until use. Mice fed the HF/HS diet or the HF/HS/HC diet are referred to as DIO or NASH mice. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Connecticut.

2.2 Plasma Chemistry and liver lipid measurements

Plasma lipids were determined enzymatically using Cholesterol Reagent (Pointe Scientific, Canton, MI) and a L-Type TG M kit (Wako Chemical, Richmond, VA) for total

cholesterol (TC) and triglyceride (TG), respectively, as we previously described [39]. Plasma alanine transaminase (ALT) activity was measured using a Liquid ALT (SGPT) Reagent set (Pointe Scientific, Canton, MI). A Liquid Glucose (Oxidase) reagent set (Pointe Scientific) was used to determine plasma glucose concentrations according to the manufacturer's protocol. Liver TC and TG were measured enzymatically from lipids extracted by Folch's methods as we previously described [40].

2. 3. Histology of liver and eAT

Formalin-fixed liver and eAT were embedded in paraffin and cut into 5 μ m sections, after which the sections were stained with hematoxylin and eosin (H&E). Tissue images were viewed at 20x magnification using an AxioCam MRc camera (Carl Zeiss Microscopy, Jena, Germany). Formalin-fixed liver samples were also stained with Gomoris trichrome for collagen and viewed at 20x magnification. Both H&E and Gomoris trichrome staining were performed at the Veterinary Medical Diagnostic Laboratory. For collagen staining in eAT, OCT-frozen eAT samples were sliced to a thickness of 10 μ m using a Leica CM3050 cryostat (Buffalo Grove, IL). The sections were stained with a sirius red/fast green collagen staining kit (Chondrex, Inc., Redmond, WA) following the manufacturer's protocol. The images were viewed at 20x magnification.

2. 4. Splenocyte isolation

Splenocytes were isolated from mice on an experimental diet as we previously described [41]. Isolated splenocytes were resuspended in RPMI-1640 media and plated at a density of 2 x

10⁶ cells/L. The cells were then stimulated by 500 ng/ml of lipopolysaccharide (LPS) for 10 h and subsequently harvested to measure the expression of inflammatory genes.

2. 5. Gene expression analysis by quantitative realtime PCR (qRT-PCR)

Total RNA was isolated from tissue samples and splenocytes using TRIzol® RNA Isolation Reagent (ThermoFisher scientific, Waltham, MA). qRT-PCR analysis was conducted as previously described using SYBR Green procedure and CFX96 RT PCR detection system (Bio-Rad, Hercules, CA) [36, 41]. Primer sequences are listed in **Supplemental Table 1**.

2. 6. Statistical Analysis

Data were expressed as mean ± SEM. One-way ANOVA and Newman-Keuls pairwise comparisons or unpaired t-test to detect statistically significant differences between groups were conducted using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). P values less than 0.05 were considered significant.

3. Results

3.1. Effect of ASTX on body and tissue weights, and plasma lipids and glucose in DIO mice

Compared with LF control, HF/HS and AHF/HS significantly increased body, liver, and adipose tissue weights as well as hepatic TC and TG levels (**Table 2**). Body weights of mice throughout the experimental period are shown in **Supplemental Figure 1**. The increase in rAT weight by HF/HS was significantly attenuated by ASTX supplementation in DIO mice.

Although HF/HS-fed mice showed significantly higher plasma TC, TG, ALT and glucose concentrations than LF group, plasma TG and ALT levels in AHF/HS-fed mice were not significantly different from those of LF control mice (**Table 3**).

3.2 Effect of ASTX on macrophage infiltration and fibrosis in eAT of DIO mice

We measured the expression of macrophage markers and phenotypes in eAT. ASTX supplementation significantly decreased the expression of F4/80 mRNA, a well-known marker of macrophages, without affecting the expression of cluster of differentiation 68 (CD68) mRNA (**Figure 1A**). The expression of CD11c and MCP-1, markers of M1 macrophages, was significantly lower in mice fed AHF/HS than HF/HS-fed mice, whereas M2 macrophage markers, including CD206, Arg-1, and IL-10, did not significantly differ between the two groups. Caspase 3 (Casp-3) expression, but not Casp-9, was significantly lower in AHF/HS than HF/HS group. In eAT of HF/HS mice, marked accumulation of macrophages with irregular-sized adipocytes were present, which was markedly attenuated in AHF/HS-fed mice (**Figure 1B**). The accumulation of macrophages was not readily noticeable in epididymal fat pad of mice fed LF diet (**Supplemental Figure 2**).

To investigate the effect of ASTX on adipose fibrosis, the expression of genes involved in fibrosis was measured in eAT. ASTX significantly decreased the expression of genes for extracellular matrix proteins such as collagen type I, α 1 (COL1A1), collagen type VI, α 1 (COL6A1) and COL6A3 (**Figure 1C**). The expression of lysyl oxidase-like 2 (LOXL2), an enzyme associated with cross-linking elastin and collagen for fibrosis, and hypoxia-inducible factor 1- α (HIF1 α) was also significantly decreased in eAT of AHF/HS group relative to HF/HS

group. Likewise, less collagen staining in eAT was observed with AHF/HS compared with HF/HS (**Figure 1D**).

3.3. Effect of ASTX on the hepatic expression of genes involved in lipid metabolism, inflammation and fibrosis in DIO mice

The hepatic expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) and low-density lipoprotein receptor (LDLR) was not significantly different between HF/HS and ASF/HS groups (**Table 4**). Fatty acid synthase (FAS) mRNA was significantly lower in AHF/HS-fed mice than HF/HS controls while stearoyl CoA desaturase 1 (SCD-1) mRNA tended to decrease with ASTX (P=0.078). No significant differences were noted in macrophage markers and markers specific for M1 or M2 macrophages between HF/HS and AHF/HS groups, except that a significant increase in Arg-1 was observed in the liver of ASTX-fed mice. However, hepatic expression of several fibrogenic genes, including lumican (LUM), COL1A1, COL6A1 and COL6A3, were significantly decreased in AHF/HS groups compared with controls.

3.4. Effect of ASTX on gene expression in the skeletal muscle and white adipose tissue of DIO mice

In the skeletal muscle, ASTX significantly increased the mRNA levels of carnitine palmitoyltransferase 1 α (CPT-1 α) and acyl-coenzyme A oxidase 1 (ACOX-1), the rate limiting enzymes for mitochondrial and peroxisomal fatty acid β -oxidation, respectively, compared with HF/HS controls (**Table 4**). Also, peroxisome proliferator-activated receptor α (PPAR α) and PPAR γ co-activator 1 α (PGC-1 α) mRNA levels were significantly higher in AHF/HS-fed mice

than HF/HS-fed mice. There were no significant differences in the genes related to lipogenesis in white adipose tissue between HF/HS and AHF/HS groups.

3.5. Effect of ASTX on inflammation and fibrosis in the eAT of a diet-induced NASH mouse model

Although feeding of HF/HS diet for 30 weeks induced drastic alterations in adipose tissue, as described above, we did not observe significant induction of inflammatory and fibrogenic genes in the liver. Previously, dietary cholesterol at 2% was shown to induce NASH in mice [42]. Therefore, we further examined if HF/HS diet with 2% cholesterol (i.e., HF/HS/HC) would induce NASH and whether ASTX supplementation at a lower level (0.015%, wt/wt) (AHF/HS/HC) would mitigate inflammation and fibrosis. In eAT, ASTX significantly decreased mRNA levels of macrophage markers, i.e., F4/80 and CD68, compared with NASH controls. Also, we observed significant decreases in M1 type macrophage markers, such as CD11c and MCP-1, whereas M2 macrophage marker, CD206, was significantly higher in AHF/HS/HC-fed mice than NASH control mice (**Figure 2A**). The expression of genes associated with fibrogenesis/fibrosis, e.g. LUM, vimentin (VIM), COL1A1, COL6A1, COL6A3, matrix metalloproteinase-2 (MMP2) and HIF1 α , was significantly lower in eAT of AHF/HS/HC group than control (**Figure 2B**).

3.6. Effect of ASTX on the features of NASH in a diet-induced NASH mouse model

Histological analysis demonstrated that the presence of microvesicular lipid droplets was noticeable in the HF/HS/HC control group compared with mice fed ASTX at 0.015% (wt/wt) in the liver. (**Figure 3A**). Furthermore, F4/80 and CD68 mRNA were significantly decreased by

ASTX with a concomitant decrease in M1 macrophage markers such as CD11c and MCP-1 (Figure 3B).

Collagen accumulation in the liver was evident in HF/HS/HC mouse livers based on Gomoris Trichrome staining, whereas minimal collagen accumulation was seen in ASTX-fed mice (Figure 3C). Similarly, the expression of hepatic fibrogenic genes, including TGF β 1, LUM, tenascin-C (TNC) and COL1A1, was significantly lower with ASTX supplementation (Figure 3D).

3.7. Effect of ASTX on pro-inflammatory gene expression in the splenocytes of DIO mice

Primary splenocytes isolated from LF, HF/HS and AHF/HS-fed mice were evaluated for their inflammatory responsiveness to LPS. The levels of TNF α mRNA were significantly lower in both unstimulated and LPS-stimulated splenocytes from AHF/HS-fed mice, compared with those from HF/HS control mice, whereas IL-6 and IL-1 β mRNA levels did not significantly differ between groups (Figure 4).

4. Discussion

Metabolic stress due to excessive fat accumulation and macrophage infiltration into the lipid-laden adipose tissue triggers chronic low-grade inflammation, which is associated with the co-morbidities of obesity such as NAFLD and type 2 diabetes [43-45]. Importantly, recent studies emphasize that not only the number of macrophages, but macrophage phenotypes play important roles in metabolic disturbances and inflammatory state in adipose tissue [19, 46]. In the present study, we evaluated the effect of ASTX on obesity-induced metabolic dysfunctions, inflammation, and fibrosis in two mouse models, i.e., DIO mice and diet-induced NASH mice.

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4 225 Although C57BL/6J mice fed a diet high in fat and sucrose with 0.2% sucrose in drinking water
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6 226 for 18 weeks displayed NASH features including fibrosis [47], our study here showed that
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8 227 C57BL/6J mice fed a HF/HS diet showed minimal increases in the expression of hepatic
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10 228 fibrogenic genes even after 30 weeks on this diet. As the addition of dietary cholesterol is known
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12 229 to induce NASH with fibrosis in C57BL/6J mice [42, 48], we formulated a HF/HS/HC diet to
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14 230 develop a mouse model of NASH with fibrosis. Cholesterol supplementation to a diet high in fat
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16 231 and sucrose induced adverse metabolic and inflammatory effects as well as fibrosis both in
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18 232 adipose tissue and the liver, which was counteracted by ASTX.
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24 233 Macrophage infiltration into adipose tissue triggers chronic inflammation by producing
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26 234 pro-inflammatory cytokines [2-4]. In the present study, we found that ASTX significantly
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28 235 reduced the expression of F4/80, a well-known marker of macrophages, in the epididymal fat
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30 236 pads compared with HF/HS controls. Consistently, the appearance of crown-like structure, a
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32 237 feature of macrophages surrounding a necrotic adipocyte [49], was markedly reduced in the eAT
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34 238 of AHF/HS mice. The result suggests that monocyte recruitment to adipose tissue is attenuated
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36 239 by ASTX. Recent studies demonstrate that local proliferation of macrophages is the primary
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38 240 source of tissue macrophages rather than recruited monocytes into mouse tissues [50, 51].
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40 241 Therefore, ASTX may also inhibit macrophage proliferation in adipose tissue. Further work is
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42 242 needed to clarify this potential action of ASTX.
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48 243 Evidence suggests that phenotypes of macrophages in lipid-laden adipose tissue are an
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50 244 important determinant of metabolic dysfunctions. The deletion of M1 marker genes, such as
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52 245 TNF α [23] and MCP-1 [24], or the ablation of CD11c⁺ cells normalized insulin sensitivity in
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54 246 DIO mice [25]. In contrast, when PPAR γ or δ was deleted selectively in macrophages, mice
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56 247 exhibited insulin resistance with reduced number and impaired function of M2 macrophages [52-
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55]. In obese mice, a phenotypic shift from an M2 state to a pro-inflammatory M1 type [19] and increased number of M1 macrophages and M1-to-M2 ratios were related to the development of insulin resistance [26]. These studies support that number as well as phenotypes of macrophages are critical determinants of local and systemic metabolic disturbances and inflammation in obesity. We found that there were significant decreases in the expression of M1 macrophage markers, i.e., CD11c and MCP-1, in eAT of DIO and NASH mice fed ASTX. Although AHF/HS-fed mice did not show significant changes in the expression of M2 macrophage markers (e.g., CD206 and Arg-1), the M2 markers were significantly increased by ~5-fold in eAT of AHF/HS/HC-fed mice. In the liver of NASH mice, we also observed that ASTX significantly decreased mRNA levels of macrophage marker F4/80 and CD68 with concomitant decreases in M1 markers (i.e., CD11c and MCP-1) and increases in M2 marker (i.e., IL-10). Our findings suggest that ASTX may exert its anti-inflammatory effect by facilitating macrophage phenotypic switch from pro-inflammatory M1 to anti-inflammatory M2. This speculation is further supported by our observation in the splenocytes isolated from mice fed either HF/HS or AHF/HS. The spleen functions as a storage site for a half of the monocytes produced by the bone marrow [56]. It has been shown that in both *apoE*^{-/-} and *Ldlr*^{-/-} mice, omega-3 fatty acids significantly reduced monocytes from splenic Ly6C (hi), classical monocytes, which actively respond to pro-inflammatory stimuli, and also decreased monocyte recruitment into atherosclerotic lesions [57]. In the present study, the splenocytes from ASTX-fed mice showed lower TNF α mRNA levels than those from HF/HS control mice in both unstimulated and LPS-stimulated splenocytes. We postulate that ASTX may render splenic monocytes to be less sensitive to LPS possibly by altering monocyte phenotypes, which is well supported by our observation that fewer M1 macrophages are present in eAT of ASTX-fed mice.

Fibrosis is known as a key element that can cause inflammation in lipid-laden adipose tissue [14, 16]. ECM proteins surrounding adipocytes mechanically support the cells. As the flexibility of ECM gives healthy expansion of adipose tissue, fibrosis limits the expansion and therefore causes inflammation and ectopic lipid accumulation [11, 58]. It is not clear whether fibrosis in adipose tissue is a cause or a consequence of inflammation in obesity [16]. HIF1 α , a transcription factor that regulates cellular adaptation to hypoxia, has been shown to play a role in triggering inflammation and fibrosis in adipose tissue as adipose tissue expansion leads to a hypoxic state and HIF1 α activation [59, 60]. In the present study, we observed that ASTX supplementation in both DIO and NASH mouse models significantly decreased HIF1 α expression and its downstream genes, such as COL1A1, COL6A1 and COL6A3, in the adipose tissue of DIO mice. As collagen accumulation is associated with adipose tissue inflammation characterized by infiltration of M1 macrophages [61, 62], the marked reduction of macrophage infiltration by ASTX, as observed here, may be due partly to the decrease in pro-fibrotic collagen accumulation in adipose tissue. In particular, COL6 is a highly expressed ECM in adipose tissue and a low expression of COL6 has been shown to decrease adipose inflammation in DIO and *ob/ob* mice [62]. Also, we found the expression of LOXL2, a major component of adipose tissue fibrosis that facilitates cross-linking of collagens, was significantly decreased by ASTX. Therefore, decreased expression of COL6A1, COL6A3 and LOXL2 by ASTX may prevent fibrosis and inflammation in adipose tissue, minimizing systemic inflammation in obesity.

Fibrosis is also one of the hallmark features of NASH. We found that ASTX significantly decreased the hepatic expression of LUM, COL6A1 and COL6A3 in DIO mice. Also, in the liver of NASH mice, ASTX significantly decreased the expression of fibrogenic genes, including transforming growth factor β 1 (TGF β 1), LUM, TNC, COL1A1, COL6A1 and COL6A3. Studies

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4 294 have shown that LUM, a leucine-rich extracellular matrix proteoglycan that maintains fibril
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6 295 stability, and COL6 are highly expressed in patients with NASH [63, 64] and liver cirrhosis [65,
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9 296 66]. The decreased fibrogenic gene expression was substantiated by reduced collagen
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11 297 accumulation in the liver of ASTX-fed NASH mice. These data indicate that ASTX exerts anti-
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14 298 fibrotic effects by inhibiting the expression of several fibrogenic genes. We recently showed that
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16 299 ASTX prevented pro-fibrogenic gene expression by inhibiting TGF β 1-induced activation of
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19 300 Smad3 pathway in LX-2 cells and also by preventing the activation of mouse primary HSCs
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21 301 [38]. Therefore, the anti-fibrotic effect of ASTX may be attributable, at least in part, to its
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24 302 inhibitory action of Smad3 signaling and HSC activation. In-depth investigation of the molecular
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26 303 mechanisms by which ASTX prevents fibrosis in the liver and adipose tissue is currently
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29 304 underway in our laboratory.

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31 305 Skeletal muscle is the primary site for utilizing fatty acids to generate energy through
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33 306 mitochondrial oxidative system. Obesity promotes intramyocellular lipid accumulation and
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36 307 mitochondrial dysfunctions, which can impair fatty acid oxidation in the skeletal muscle [67].
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38 308 Reduced oxidative capacity of skeletal muscle is primarily due to reduced activity of enzymes
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41 309 involved in fatty acid oxidation, such as CPT-1 α and ACOX-1 [68]. The activation of their
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44 310 transcriptional regulator, PPAR α , is known to improve mitochondrial fat oxidation and insulin
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46 311 sensitivity in obesity [69, 70]. PGC-1 α plays an important role in regulating the transcriptional
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49 312 activity of PPAR α as a component of a coactivator complex to increase mitochondrial and
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51 313 peroxisomal fatty acid oxidation in skeletal muscle [71-76]. In addition, as ASTX is suggested to
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54 314 function as PPAR α agonist [77], it may directly activate PPAR α . In the present study using DIO
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56 315 mice, we found that ASTX supplementation significantly increased the expression of CPT-1 α
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59 316 and ACOX-1 in the skeletal muscle as well as their transcriptional regulator PPAR α [78, 79].
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Furthermore, the expression of PGC-1 α was induced by ~3-fold in the ASTX-fed mice, suggesting that the induction of PGC-1 α by ASTX may explain the marked increases in CPT-1 α and ACOX-1 expression despite the significant, but modest, increase in PPAR α by ASTX. It should be noted that despite the marked inhibition of macrophage infiltration by ASTX in adipose tissue, we did not observe significant changes in the expression of lipogenic genes in eAT of DIO mice fed ASTX. Mitochondrial dysfunction contributes to an array of human pathological conditions, leading to the accumulation of lipid and reactive oxygen species (ROS) [80, 81], which in turn trigger oxidative stress and inflammation [82-84]. In contrast, enhanced mitochondrial function can reduce fat accumulation and ROS production, and prevent the development of insulin resistance and chronic inflammation by efficiently oxidizing energy substrates [85]. Therefore, it is probable that the primary action of ASTX in the skeletal muscle is to enhance the muscular capacity of fatty acid oxidation, thereby preventing obesity-associated inflammation in adipose tissue.

In conclusion, the present study showed that ASTX prevents obesity-induced metabolic abnormalities, inflammation and fibrosis in adipose tissue and the liver. Also, our results suggest that ASTX may enhance the oxidative capacity of skeletal muscle to utilize excess energy. Further works are needed to explore how ASTX enhances energy utilization in the skeletal muscle and decreases M1 macrophage infiltration and fibrosis in adipose tissue and the liver. The present findings strongly support that ASTX supplementation may be used as an effective dietary component to prevent obesity-associated metabolic disturbances, inflammation, and fibrosis.

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9 342 Harness, and Y. Park conducted experiments; Sung I. Koo contributed to data interpretation and
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Figure legends

Figure 1. The effect of ASTX on inflammation and fibrosis in eAT of DIO mice fed a HF/HS control or a HF/HS supplemented with 0.03% ASTX (w/w) (AHF/HS) for 30 weeks. **A.** The expression of macrophage markers in eAT. Data are expressed as relative expression to control. n=9 for each group. **B.** Representative sections of eAT stained with H&E. Arrows indicate crown-like structures. **C.** The expression of fibrogenic genes in eAT. Data are expressed as relative expression to control. n=9 for each group. **D.** Representative sections of eAT stained with picrosirius red for collagen. Red and yellow indicate collagen and muscle fibers, respectively.

Figure 2. The effect of ASTX on the expression of inflammatory and fibrogenic genes in eAT of NASH mice fed a HF/HS/HC control or the diet supplemented with 0.015% ASTX (w/w) (AHF/HS/HC) for 18 weeks. **A.** mRNA levels of markers of macrophages, M1 type and M2 type. **B.** mRNA levels of fibrogenic genes. Data are expressed as relative expression to control. n=3 for each group.

Figure 3. The effect of ASTX in inflammation and fibrosis in the liver of NASH mice fed a HF/HS/HC control or a HF/HS/HC supplemented with 0.015% ASTX (w/w) (AHF/HS) for 18 weeks. **A.** Representative liver sections stained with H&E. **B.** mRNA levels of markers of macrophages, M1 and M2 phenotype. Data are expressed as relative expression to control. n=3 for each group. **C.** Representative liver sections stained with Gomoris Trichrome. Blue indicates collagen. **D.** mRNA levels of fibrogenic genes. Data are expressed as relative expression to control. n=3 for each group.

Figure 4. Inflammatory gene expression in the splenocytes isolated from C57BL/6J mice fed a HF/HS control or a HF/HS supplemented with 0.03% (w/w) ASTX (AHF/HS) for 30 weeks. Isolated splenocytes were activated with LPS (500 ng/ml) for 10 h, after which qRT-PCR was conducted for gene analysis. Data are expressed as relative expression to control. n=7 for LF, n=9 for HF/HS and AHF/HS.

Table 1. Composition of the experimental diets

Ingredient	LF	HF/HS	AHF/HS ¹
		<i>g/kg</i>	
Cornstarch	450.0	0.0	0.0
Dextrinized cornstarch	85.0	0.0	0.0
Sucrose	100.0	350.0	350.0
Casein	200.0	200.0	200.0
L-Cystine	3.0	3.0	3.0
Lard	20.0	305.0	305.0
Soybean oil	40.0	40.0	34.0
Insoluble fiber ²	50.0	50.0	50.0
AIN-93 mineral mix	40.0	40.0	40.0
AIN-93 vitamin mix	10.0	10.0	10.0
Choline bitartrate	2.0	2.0	2.0
t-Butylhydroquinone	0.008	0.008	0.008
AstaREAL [®] Oil 50F ³	0.0	0.0	6.0

¹ AHF/HS diet containing 0.03% ASTX by weight.

² Solka-Floc cellulose

³ AstaREAL[®] Oil 50F contains 5% ASTX.

Table 2. Body weight, liver weight and lipid contents and adipose weight of C57BL/6J mice fed HF/HS diet supplemented with ASTX for 30 weeks^{1,2}

	Initial body Weight (g)	Final body Weight (g)	Liver weight (g)	Liver lipid contents (mg/g wet wt)		Epididymal Adipose weight (mg/g body wt)	Retroperitoneal Adipose weight (mg/g body wt)
				TC	TG		
LF	23.3 ± 0.5	45.0 ± 1.2 ^b	1.66 ± 0.16 ^b	1.90 ± 0.31 ^b	148.0 ± 12.0 ^b	1.67 ± 0.08 ^a	0.68 ± 0.07 ^c
HF/HS	23.3 ± 0.6	54.7 ± 1.0 ^a	3.04 ± 0.15 ^a	2.58 ± 0.30 ^a	202.9 ± 11.3 ^a	1.35 ± 0.07 ^b	1.29 ± 0.06 ^a
AHF/HS	23.3 ± 0.5	52.1 ± 1.2 ^a	2.93 ± 0.24 ^a	2.46 ± 0.18 ^a	215.4 ± 13.1 ^a	1.23 ± 0.08 ^b	1.05 ± 0.05 ^b

¹Data represent mean ± SEM (n = 7 for LF; n = 9 for HF/HS and AHF/HS)

²Values with a different letter in a column are significantly different (P< 0.05)

Table 3. Plasma chemistry of C57BL/6J mice fed a LF control, a HF/HS control or a HF/HS diet supplemented with 0.03% ASTX (w/w) (AHF/HS) for 30 weeks^{1,2}

	TC (mg/dL)	TG (mg/dL)	ALT (IU/L)	Glucose (mmol/L)
LF	114.0 ± 15.3 ^b	31.0 ± 1.4 ^b	22.2 ± 4.5 ^b	9.11 ± 0.38 ^b
HF/HS	262.8 ± 17.5 ^a	41.9 ± 2.9 ^a	43.6 ± 4.5 ^a	16.82 ± 0.36 ^a
AHF/HS	216.1 ± 21.3 ^a	35.6 ± 2.0 ^{ab}	34.2 ± 3.1 ^{ab}	15.91 ± 0.23 ^a

¹Data represent mean ± SEM (n = 7 for LF; n = 9 for HF/HS and AHF/HS)

²Values with a different letter in a column are significantly different (P< 0.05)

Table 4. Gene expressions in liver, muscle and adipose tissues of C57BL/6J mice fed HF/HS diet supplemented with ASTX for 30 weeks¹

Tissue	Function	Gene	HF/HS	AHF/HS
<i>Relative expression</i>				
Liver	Cholesterol metabolism	HMGR	1.40 ± 0.41	0.81 ± 0.19
		LDLR	1.22 ± 0.27	0.84 ± 0.12
	Lipogenesis	FAS	1.68 ± 0.42	0.80 ± 0.20
		SCD-1	1.07 ± 0.14	0.62 ± 0.11*
	Inflammation	SREBP-1c	1.15 ± 0.18	0.84 ± 0.15
		F4/80	1.12 ± 0.20	0.66 ± 0.09
		CD68	1.24 ± 0.27	1.27 ± 0.22
		CD11c	1.18 ± 0.25	0.86 ± 0.09
		MCP-1	1.26 ± 0.24	0.95 ± 0.27
		CD206	1.06 ± 0.10	0.77 ± 0.10
		Arg-1	1.02 ± 0.74	27.3 ± 6.80*
	Fibrogenesis	TLR4	1.27 ± 0.25	0.74 ± 0.15
		αSMA	1.21 ± 0.29	1.10 ± 0.14
		TGFβ1	1.30 ± 0.31	0.86 ± 0.18
		LUM	1.08 ± 0.16	0.70 ± 0.07*
		TNC	1.15 ± 0.19	0.80 ± 0.12
		VIM	1.06 ± 0.13	1.08 ± 0.11
		COL1A1	1.34 ± 0.33	1.06 ± 0.24*
		COL6A1	1.11 ± 0.15	0.67 ± 0.09*
		COL6A3	1.39 ± 0.38	0.54 ± 0.11*
		MMP2	1.51 ± 0.43	0.81 ± 0.30
Skeletal muscle	Fatty acid metabolism	CPT-1α	1.00 ± 0.22	4.82 ± 1.54*
		CPT-1β	1.74 ± 0.64	2.44 ± 0.78
		ACOX-1	1.03 ± 0.10	1.35 ± 0.07*
		PPARα	1.04 ± 0.10	1.31 ± 0.07*
	Uncoupling	UCP-2	1.08 ± 0.15	1.61 ± 0.32
		UCP-3	1.17 ± 0.21	1.53 ± 0.58
	Mitochondria biogenesis	PGC-1α	0.91 ± 0.18	2.66 ± 0.75*
White adipose ²	Fatty acid metabolism	FAS	0.92 ± 0.18	1.41 ± 0.38
		SCD-1	1.04 ± 0.10	0.75 ± 0.09
		SREBP-1c	1.10 ± 0.16	1.09 ± 0.14
		PPARγ	1.04 ± 0.1	0.85 ± 0.13

¹ Data represent mean ± SEM (n=9).

² Epididymal adipose tissue

*, Significantly different from HF/HS in a row (P< 0.05).

Figure(s)
Figure 1

Figure 2

Figure 3

Figure 4