

Astaxanthin lowers plasma TAG concentrations and increases hepatic antioxidant gene expression in diet-induced obesity mice

Yue Yang, Tho X. Pham, Casey J. Wegner, Bohkyung Kim, Chai Siah Ku, Young-Ki Park and Ji-Young Lee*

Department of Nutritional Sciences, University of Connecticut, Storrs, CT 06269, USA

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is significantly associated with hyperlipidaemia and oxidative stress. We have previously reported that astaxanthin (ASTX), a xanthophyll carotenoid, lowers plasma total cholesterol and TAG concentrations in *apoE* knockout mice. To investigate whether ASTX supplementation can prevent the development of NAFLD in obesity, male C57BL/6J mice (*n* 8 per group) were fed a high-fat diet (35%, w/w) supplemented with 0, 0.003, 0.01 or 0.03% of ASTX (w/w) for 12 weeks. The 0.03% ASTX-supplemented group, but not the other groups, exhibited a significant decrease in plasma TAG concentrations, suggesting that ASTX at a 0.03% supplementation dosage exerts a hypotriacylglycerolaemic effect. Although there was an increase in the mRNA expression of fatty acid synthase and diglyceride acyltransferase 2, the mRNA levels of acyl-CoA oxidase 1, a critical enzyme in peroxisomal fatty acid β -oxidation, exhibited an increase in the 0.03% ASTX-supplemented group. There was a decrease in plasma alanine transaminase (ALT) and aspartate transaminase (AST) concentrations in the 0.03% ASTX-supplemented group. There was a significant increase in the hepatic mRNA expression of nuclear factor erythroid 2-related factor 2 and its downstream genes, which are critical for endogenous antioxidant mechanism, in the 0.03% ASTX-supplemented group. Furthermore, there was a significant decrease in the mRNA abundance of *IL-6* in the primary splenocytes isolated from the 0.03% ASTX-supplemented group upon lipopolysaccharide (LPS) stimulation when compared with that in the splenocytes isolated from the control group. In conclusion, ASTX supplementation lowered the plasma concentrations of TAG, ALT and AST, increased the hepatic expression of endogenous antioxidant genes, and rendered splenocytes less sensitive to LPS stimulation. Therefore, ASTX may prevent obesity-associated metabolic disturbances and inflammation.

Key words: Astaxanthin: Non-alcoholic fatty liver disease: Hypotriacylglycerolaemia: Antioxidants: Diet-induced obesity mice

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver diseases in Western countries⁽¹⁾. It is characterised by excessive fat accumulation over 5–10% of the total liver weight in patients with little or no history of alcohol consumption⁽²⁾. NAFLD is closely associated with increased fat deposition in the liver due to elevated lipolysis in the adipose tissue of obese subjects⁽³⁾. In approximately 10–25% of NAFLD patients, the disease progresses to non-alcoholic steatohepatitis (NASH), which is characterised by hepatocyte damage, inflammatory cell infiltration and fibrosis^(2,4). NASH can further progress to cirrhosis and hepatocellular carcinoma⁽⁵⁾, increasing mortality in patients with liver diseases⁽⁶⁾. Currently, there is no effective therapy available for NASH patients⁽⁷⁾. The challenge to the development of NASH therapy is largely the complexity of the disease, which is closely related to visceral obesity, dyslipidaemia, insulin resistance and hyperglycaemia⁽³⁾.

Astaxanthin (ASTX) is a xanthophyll carotenoid that is mainly present in marine animals, such as salmon and crustaceans^(8,9). ASTX acts a potent antioxidant by functioning as a powerful reactive oxygen species quencher as well as a free radical scavenger⁽¹⁰⁾. It is known that ASTX can also scavenge peroxy radicals and destroy peroxides, thereby protecting fatty acids and biological membranes from lipid peroxidation^(11,12). Studies have shown that ASTX has a protective effect against oxidative stress, inflammation and metabolic disorders, such as type 2 diabetes and CVD, in animals^(13–15) and humans^(16,17). In our previous study, *apoE* knockout (*apoE*^{-/-}) mice fed an atherogenic diet supplemented with ASTX exhibited decreases in the plasma concentrations of total cholesterol (TC) and TAG⁽¹³⁾. Furthermore, ASTX also increased the expression of nuclear factor erythroid 2-related factor 2 (NRF-2)-responsive antioxidant genes and decreased the concentrations of an oxidative

Abbreviations: ACOX-1, acyl-CoA oxidase 1; ALT, alanine transaminase; AST, aspartate transaminase; ASTX, astaxanthin; DIO, diet-induced obesity; HF, high fat; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LPS, lipopolysaccharide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NRF-2, nuclear factor erythroid 2-related factor 2; SREBP, sterol regulatory element-binding protein; TC, total cholesterol; TGF β 1, transforming growth factor- β 1.

* **Corresponding author:** Dr J.-Y. Lee, fax +1 860 486 3674, email ji-young.lee@uconn.edu

stress marker, glutathione disulphide, in the liver⁽¹³⁾. The study demonstrated that ASTX supplementation improves lipid metabolism as well as enhances antioxidant defence mechanisms in *apoE*^{-/-} mice, supporting that ASTX may prevent the development of NAFLD.

In the present study, we aimed to determine the effective dietary supplementation dosage of ASTX at which it could counteract metabolic dysfunctions as well as to evaluate whether ASTX supplementation can improve metabolic and inflammatory parameters that are disturbed by a high-fat (HF) diet in diet-induced obesity (DIO) mice.

Materials and methods

Animal feeding and care

Male C57BL/6J mice aged 6 weeks were obtained from Jackson Laboratory. After 1 week of acclimation period, mice were randomly assigned to one of the following four groups: group fed a HF control diet (35%, w/w); group fed a HF diet supplemented with 0.003% of ASTX; group fed a HF diet supplemented with 0.01% of ASTX; group fed a HF diet supplemented with 0.03% of ASTX (by weight) (*n* 8 per group). AstaREAL[®] L10, an extract from the microalgae *Haematococcus pluvialis* containing 10% of ASTX and approximately 90% of TAG, was kindly provided by Fuji Chemical Industry Corporation Limited. Based on body surface normalisation to a 70 kg individual⁽¹⁸⁾, ASTX supplementation dosages of 0.003, 0.01 and 0.03% in mice would be equivalent to approximately 20, 66 and 200 mg/d in humans. Diet composition is given in Table 1. Mice were housed in polycarbonate cages under a 12h light–12h dark cycle with free access to food and water for 12 weeks. Body weight and food intake were measured weekly. At the end of 12 weeks of feeding, mice were anaesthetised with ketamine/xylazine (120/6 mg/kg; Henry Schein Animal Health) after 8 h of fasting and then killed by cardiac puncture and cervical dislocation. Blood samples were collected in EDTA-containing vacutainers (BD Vacutainer[®]) and centrifuged at 1500 g at 4°C for 15 min

Table 1. Composition of the high-fat (HF) control diet and the HF diets containing astaxanthin (ASTX)*

Composition (g/kg)	Control†	ASTX		
		0.003%	0.01%	0.03%
Dextrinised maize starch	162.6	162.6	162.6	162.6
Sucrose	89.5	89.5	89.5	89.5
Casein	260.1	260.1	260.1	260.1
L-Cystine	3.9	3.9	3.9	3.9
Coconut oil	318.7	318.7	318.7	318.7
Soyabean oil	32.5	32.2	31.5	29.5
Insoluble fibre	65.0	65.0	65.0	65.0
AIN-93 mineral mix	52.0	52.0	52.0	52.0
AIN-93 vitamin mix	13.0	13.0	13.0	13.0
Choline bitartrate	2.6	2.6	2.6	2.6
<i>tert</i> -Butylhydroquinone	0.01	0.01	0.01	0.01
AstaREAL [®] L10	0	0.3	1.0	3.0
Total	1000.0	1000.0	1000.0	1000.0

* ASTX supplementation dosages by weight.

† The HF control diet contained 35% fat by weight.

to remove erythrocytes. Liver and epididymal and retroperitoneal adipose tissue samples were snap-frozen in liquid N₂ and stored at –80°C until use. A small portion of the tissue samples was fixed in 10% formalin for histological analysis. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Connecticut.

Plasma chemistry and hepatic lipid quantification

Lipid from liver samples was extracted using the Folch method as described previously⁽¹⁹⁾. Plasma and hepatic lipids were quantified using enzymatic assays for TC, TAG, and HDL-cholesterol, and plasma glucose was quantified using the Liquid Glucose (Oxidase) Reagent Set (Pointe Scientific, Inc.)⁽²⁰⁾. Plasma insulin was quantified using ELISA (Alpco) according to the manufacturer's instructions. Plasma alanine transaminase (ALT) and aspartate transaminase (AST) were quantified using the Cholestech LDX System (Cholestech Corporation).

Gene expression analysis

Total RNA was isolated from liver and adipose tissue samples as described previously using TRIzol Reagent (Invitrogen)⁽²¹⁾. Quantitative real-time PCR analysis was carried out as described previously using the SYBR Green procedure and CFX96 Touch[™] Real-Time PCR Detection System (Bio-Rad)⁽²²⁾. Primer sequences were designed according to the GenBank database using the Beacon Designer software (PREMIER Biosoft), and primer sequences are available upon request. Ribosomal protein, large, P0 was used as an internal control for data normalisation.

Primary splenocyte isolation and treatment

Primary splenocytes were isolated from freshly isolated spleens as described previously⁽²³⁾. The splenocytes were resuspended in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and were then stimulated with 100 ng/ml lipopolysaccharide (LPS) for 18 h for gene expression analysis.

Histological evaluation of liver samples

Formalin-fixed liver samples were sliced into 5 µm-thick sections and stained with haematoxylin and eosin by the Connecticut Veterinary Medical Diagnostic Laboratory. The stained tissue sections were examined using a Zeiss Axio Observer.A1 microscope (Carl Zeiss Microscopy), and images were obtained at 20 × magnification with an AxioCam MRC camera (Carl Zeiss Microscopy).

Statistical analysis

One-way ANOVA or Student's unpaired *t* test was carried out using GraphPad Prism 6 (GraphPad Software) to identify statistically significant differences between the groups with a two-tailed level of significance at *P* < 0.05. Normality and equity of variance were tested using D'Agostino–Pearson

Table 2. Body and tissue weights and plasma insulin and glucose concentrations of C57BL/6J mice fed a high-fat (HF) control diet or a HF diet supplemented with 0.03% (w/w) of astaxanthin (ASTX) for 12 weeks (Mean values with their standard errors; $n=5-8$)

	Control		0.03% ASTX	
	Mean	SEM	Mean	SEM
Body weight (g)	39.3	2.43	41.0	1.67
Total weight gain (g)	15.9	2.00	18.4	1.44
Epididymal adipose tissue weight (g)	1.80	0.23	2.62*	0.18
Retroperitoneal adipose tissue weight (g)	0.50	0.06	0.67	0.06
Plasma fasting glucose concentration (mmol/l)	17.9	2.67	19.8	1.13
Plasma fasting insulin concentration (pmol/l)	172	6.49	177	10.6

* Mean value was significantly different from that of the control group ($P<0.05$).

omnibus normality test and Bartlett's test, respectively. Data are expressed as means with their standard errors.

Results

Body and tissue weights and plasma chemistry

There were no significant differences in food intake (data not shown), body weight, body weight gain and retroperitoneal adipose tissue weight between the control and 0.03% ASTX-supplemented groups (Table 2). However, the 0.03% ASTX-supplemented group exhibited a significantly higher epididymal adipose tissue weight when compared with the control group. There were no significant differences in plasma glucose and insulin concentrations between the control and 0.03% ASTX-supplemented groups.

Hypotriacylglycerolaemic effect of 0.03% astaxanthin supplementation in diet-induced obesity mice

We have previously reported that 0.03% ASTX supplementation lowers plasma TC and TAG concentrations in

apoE^{-/-} mice fed an atherogenic diet⁽¹³⁾. To determine the effective supplementation dosage of ASTX at which it could lower blood lipid concentrations in obesity, mice were fed a HF diet supplemented with 0.003, 0.01 or 0.03% of ASTX (w/w). The 0.03% ASTX-supplemented group, but not the 0.003 and 0.01% ASTX-supplemented groups, exhibited significantly lower plasma TAG concentrations when compared with the control group (Fig. 1(a)). There were no significant differences in plasma TC and HDL-cholesterol concentrations between the control and 0.03% ASTX-supplemented groups (Fig. 1(b)). These results suggest that ASTX supplementation at a dosage of 0.03% is required to elicit a TAG-lowering effect in DIO mice. Therefore, only the 0.03% ASTX-supplemented group was used for subsequent analyses to compare with the control group.

Effect of 0.03% astaxanthin supplementation on hepatic lipid concentrations and damage

There were no significant differences in liver weight and hepatic TAG and TC concentrations between the control and 0.03% ASTX-supplemented groups (Fig. 2(a)). Consistently, histological analysis of haematoxylin and eosin-stained liver sections showed no noticeable difference in lipid accumulation between the two groups (Fig. 2(b)). However, a trend towards lower plasma ALT concentrations ($P=0.071$) and significantly lower plasma AST concentrations ($P=0.015$) was observed in the 0.03% ASTX-supplemented group when compared with the control group (Fig. 2(c)).

Effect of astaxanthin supplementation on the hepatic expression of lipogenic, cholesterogenic, inflammatory and fibrogenic genes

To gain mechanistic insights into the TAG-lowering effect of ASTX, the expression of genes involved in lipogenesis and fatty acid β -oxidation was measured in the liver. There were no differences in the mRNA abundance of sterol regulatory element-binding protein (*SREBP*)-1c, a key transcription

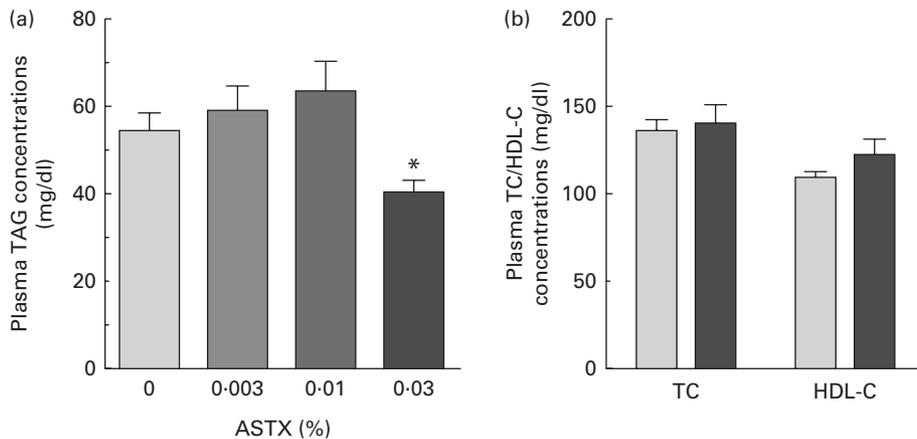


Fig. 1. Plasma lipid profiles. Male C57BL/6J mice were fed a high-fat (HF) control diet (□) or a HF diet supplemented with 0.003, 0.01 or 0.03% of astaxanthin (■) by weight for 12 weeks. (a) Plasma concentrations of TAG and (b) total cholesterol (TC) and HDL-cholesterol (HDL-C). Values are means, with their standard errors represented by vertical bars ($n=5-8$). * Mean value was significantly different from that of the control group ($P<0.05$). To convert TAG in mg/dl to mmol/l, multiply by 0.01129. To convert cholesterol in mg/dl to mmol/l, multiply by 0.02586.

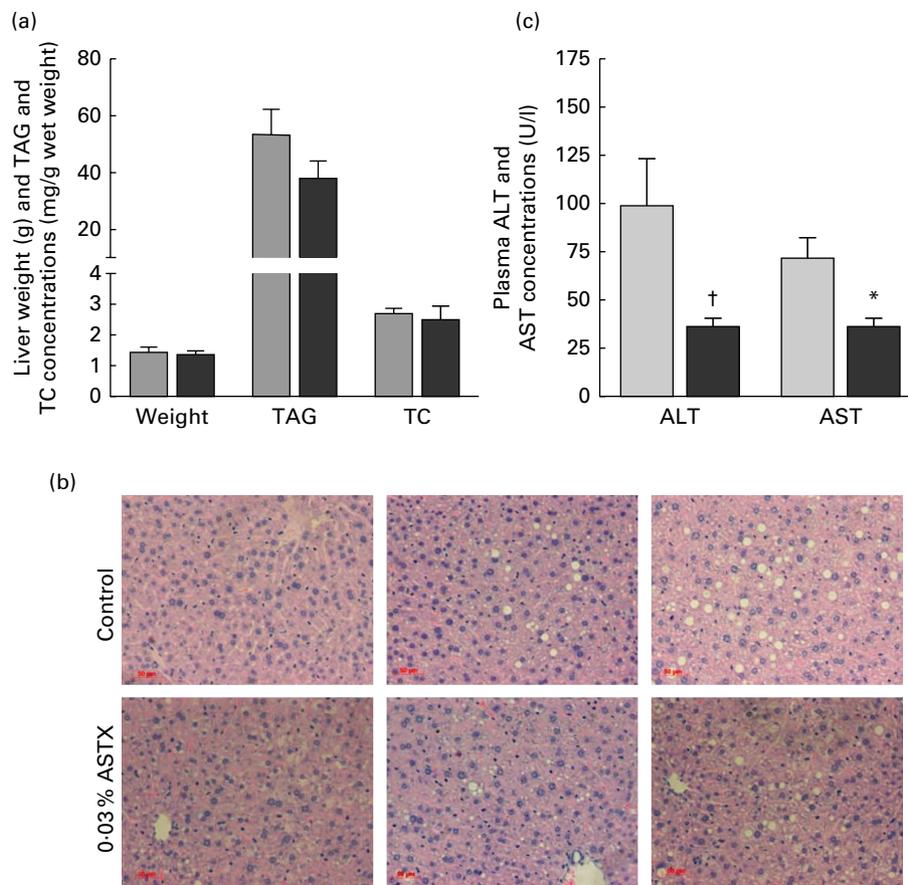


Fig. 2. (a) Liver weight and TAG and total cholesterol (TC) concentrations. (b) Representative liver sections stained with haematoxylin and eosin at 20× magnification. (c) Plasma alanine transaminase (ALT) and aspartate transaminase (AST) concentrations. Male C57BL/6J mice were fed a high-fat (HF) control diet (□) or a HF diet supplemented with 0.03% of astaxanthin (ASTX, ■) by weight for 12 weeks. Values are means, with their standard errors represented by vertical bars (n 5–8). * Mean value was significantly different from that of the control group ($P < 0.05$). † Mean value was tended to be decreased from that of control group ($P < 0.05$).

factor in lipogenesis, and stearoyl-CoA desaturase 1 between the control and 0.03% ASTX-supplemented groups, while there was a significant increase in the mRNA abundance of fatty acid synthase and diglyceride acyltransferase 2 in ASTX-supplemented mice than in control mice (Table 3). In contrast to lipogenic genes, there was a significant increase in the mRNA expression of acyl-CoA oxidase 1 (*ACOX-1*), the rate-limiting enzyme in the peroxisomal fatty acid β -oxidation, in the 0.03% ASTX-supplemented group, while there was no change in the mRNA expression of carnitine palmitoyltransferase-1 α , the rate-limiting enzyme in mitochondrial fatty acid β -oxidation. Although there were no significant differences in plasma TC and HDL-cholesterol concentrations between the groups, marginal but significant increases in the hepatic expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) and LDL receptor were observed in the 0.03% ASTX-supplemented group with no difference being observed in that of *SREBP-2*. There were no significant differences in the expression of genes involved in HDL metabolism, including scavenger receptor class B, type 1 and apoA-I, between the control and 0.03% ASTX-supplemented groups.

Inflammation with increased macrophage infiltration and elevated fibrogenesis in the liver is a major marker of NASH⁽²⁴⁾. The mRNA expression of α -smooth muscle actin and procollagen type I, α 1, which are markers of fibrosis, was not altered in the 0.03% ASTX-supplemented group, while that of transforming growth factor- β 1 (*TGF β 1*), a potent profibrogenic cytokine, was significantly increased (Table 3). There was no change in the mRNA expression of *F4/80*, a macrophage marker, in ASTX-supplemented mice.

Effect of astaxanthin supplementation on the expression of nuclear factor erythroid 2-related factor 2 and its target genes in the liver

As ASTX is a well-known antioxidant, we examined whether ASTX supplementation can alter a NRF2-mediated endogenous antioxidant defence system. There was a significant increase in the mRNA expression of *NRF-2* as well as its target genes, including superoxide dismutase 1, glutamate-cysteine ligase regulatory subunit and glutathione peroxidase 1, in the liver of the 0.03% ASTX-supplemented group than in the control group (Fig. 3).

Table 3. Hepatic and adipose tissue gene expression of C57BL/6J mice fed a high-fat (HF) control diet or a HF supplemented with 0.03% (w/w) of astaxanthin (ASTX) for 12 weeks (Mean values with their standard errors; n 5–8)

Function	Gene	Control		0.03% ASTX		<i>P</i>
		Mean	SEM	Mean	SEM	
Liver						
Lipogenesis	<i>SREBP-1c</i>	1.07	0.15	1.58	0.25	0.092
	<i>FAS</i>	1.06	0.14	1.59	0.19	0.040
	<i>SCD-1</i>	1.31	0.28	1.53	0.43	0.662
	<i>DGAT-2</i>	1.16	0.19	2.28	0.08	0.003
Fatty acid oxidation	<i>ACOX-1</i>	1.02	0.07	1.42	0.09	0.009
	<i>CPT-1α</i>	1.09	0.17	1.23	0.12	0.583
Cholesterol metabolism	<i>SREBP-2</i>	1.11	0.19	0.92	0.18	0.490
	<i>HMGCR</i>	1.06	0.14	1.59	0.19	0.040
	<i>LDLR</i>	1.13	0.22	2.04	0.40	0.048
HDL metabolism	<i>SR-B1</i>	1.10	0.17	1.45	0.36	0.346
	<i>ApoA-I</i>	1.01	0.11	1.28	0.26	0.468
Inflammation and fibrosis	α - <i>SMA</i>	1.08	0.18	0.63	0.18	0.110
	<i>Col1A1</i>	1.14	0.20	0.83	0.09	0.332
	<i>TGFβ1</i>	1.09	0.17	4.50	1.03	0.002
	<i>F4/80</i>	1.04	0.23	1.25	0.26	0.550
Epididymal adipose tissue						
Lipogenesis	<i>SREBP-1c</i>	1.10	0.20	1.42	0.26	0.338
	<i>FAS</i>	1.21	0.23	1.34	0.24	0.716
Inflammation	<i>TNFα</i>	1.74	0.56	1.52	0.44	0.789
	<i>F4/80</i>	2.61	1.08	0.70	0.13	0.250

SREBP, sterol regulatory element-binding protein; *FAS*, fatty acid synthase; *SCD-1*, stearoyl-CoA desaturase 1; *DGAT-2*, diglyceride acyltransferase 2; *ACOX-1*, acyl-CoA oxidase 1; *CPT-1 α* , carnitine palmitoyltransferase-1 α ; *HMGCR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *LDLR*, LDL receptor; *SR-B1*, scavenger receptor class B, type 1; α -*SMA*, α -smooth muscle actin; *Col1A1*, procollagen type I, α 1; *TGF β 1*, transforming growth factor- β 1.

Effect of 0.03% astaxanthin supplementation on the inflammatory responses of splenocytes

It has been suggested that the spleen functions as a reservoir of monocytes⁽²⁵⁾. To determine whether ASTX supplementation alters the inflammatory response of splenocytes, the primary cells isolated from control and 0.03% ASTX-supplemented mice were stimulated with LPS. There was a significant decrease in the mRNA abundance of *IL-6* in splenocytes isolated from 0.03% ASTX-supplemented mice ($P=0.038$) when compared

with that in splenocytes isolated from control mice, while there were no differences in the mRNA abundance of *TNF α* between the groups (Fig. 4).

Discussion

Lipotoxicity, oxidative stress and inflammation are commonly identified as key players in the pathogenesis of NASH^(26–28). Obesity is also a major risk factor for NAFLD and NASH development⁽²⁹⁾. In obesity, fatty acid flux to the liver is increased

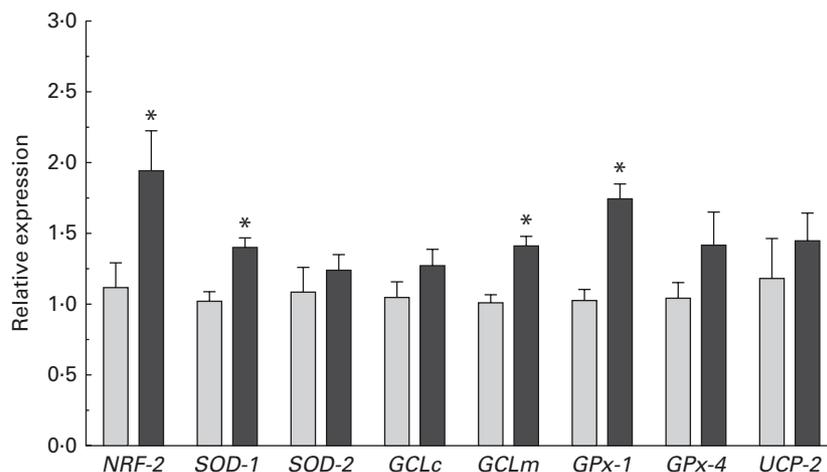


Fig. 3. Hepatic mRNA expression of antioxidant genes. Male C57BL/6J mice were fed a high-fat (HF) control diet (□) or a HF diet supplemented with 0.03% of astaxanthin (■) by weight for 12 weeks. Values are means, with their standard errors represented by vertical bars (n 8). * Mean value was significantly different from that of the control group ($P<0.05$). *NRF-2*, nuclear factor erythroid 2-related factor 2; *SOD*, superoxide dismutase; *GCLc*, glutamate–cysteine ligase, catalytic subunit; *GCLm*, glutamate–cysteine ligase regulatory subunit; *GPx*, glutathione peroxidase; *UCP-2*, uncoupling protein 2.

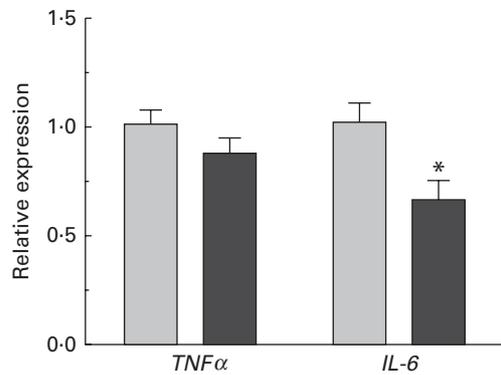


Fig. 4. Inflammatory response of splenocytes. Splenocytes were isolated from male C57BL/6J mice fed a high-fat (HF) control diet (□) or a HF diet supplemented with 0.03% of astaxanthin (■) by weight for 12 weeks and stimulated with lipopolysaccharide (100 ng/ml) for 18 h for quantitative real-time PCR analysis. Values are means, with their standard errors represented by vertical bars (n 8). * Mean value was significantly different from that of the control group ($P < 0.05$).

due to elevated lipolysis in hypertrophied adipocytes, leading to the accumulation of excessive TAG, oxidative stress, inflammation and insulin resistance in the liver. Insulin resistance promotes *de novo* lipogenesis and gluconeogenesis, further contributing to the development of dyslipidaemia, hyperglycaemia and other metabolic disorders, such as type 2 diabetes and CVD⁽³⁰⁾. The goal of the present study was to determine the effective supplementation dosage of ASTX at which it could counteract metabolic dysfunctions, oxidative stress and inflammation induced by a HF diet in DIO mice. We found that ASTX at a dosage of 0.03% exerted a TAG-lowering effect, increased hepatic expression of endogenous antioxidant genes with a concomitant decrease in steatosis, and attenuated the inflammatory response of splenocytes to LPS in DIO mice. In addition, it should be noted that ASTX lowered plasma ALT and AST concentrations in DIO mice. ALT is predominately found in the liver, whereas AST is present in the liver, cardiac and skeletal muscle, kidneys, brain and erythrocytes⁽³¹⁾. Therefore, the reduction in plasma ALT and AST concentrations by ASTX suggests that it may prevent not only liver damage, but also potential HF-induced tissue damage in other organs.

Hypertriacylglycerolaemia, a common lipid abnormality in obese patients, is a major contributor of the development of insulin resistance⁽³²⁾, type 2 diabetes⁽³³⁾, CVD⁽³⁴⁾ and NAFLD⁽³⁵⁾. Hypertriacylglycerolaemia in obesity primarily occurs due to increased hepatic *de novo* lipogenesis consequent to elevated NEFA flux into the liver from dysfunctional adipocytes⁽³⁰⁾. Consistent with our previous observation that 0.03% ASTX supplementation significantly lowers plasma TAG concentrations in *apoE*^{-/-} mice⁽¹³⁾, ASTX at the same supplementation dosage also decreased plasma TAG concentrations in DIO mice in the present study. A hypo-triacylglycerolaemic effect of ASTX has also been reported in humans⁽¹⁷⁾. In both *apoE*^{-/-} and DIO mice, we observed a significant increase in the hepatic mRNA expression of *ACOX-1*, while the expression of lipogenic genes was not altered or increased by ASTX supplementation. Hepatic TAG

concentrations in 0.03% ASTX-supplemented mice were lower by approximately 30% when compared with those in control mice, although they did not reach statistical significance. Therefore, it can be presumed that elevated peroxisomal fatty acid oxidation due to increased *ACOX-1* expression induced by ASTX may be responsible for the lipid-lowering effect of ASTX. The potential effect of ASTX on fatty acid β -oxidation is currently being investigated in our laboratory.

Hypertriacylglycerolaemia in obesity is frequently accompanied by other dyslipidaemic conditions, such as high TC and LDL-cholesterol concentrations⁽³³⁾, increasing CVD risk in obese population⁽³⁶⁾. We have previously reported that 0.03% ASTX supplementation exerts a cholesterol-lowering effect in *apoE*^{-/-} mice fed a HF/high cholesterol diet⁽¹³⁾. In the present study, although ASTX supplementation significantly increased the hepatic mRNA expression of LDL receptor and *HMGR*, two of the most important cholesterol regulatory genes under the transcription regulation of SREBP-2, we did not observe any changes in plasma and hepatic TC concentrations induced by ASTX supplementation. This discrepancy in findings with regard to the cholesterol-lowering effect of ASTX between our previous study and the present study may lie in the different animal models, i.e. *apoE*^{-/-} *v.* DIO mice, as well as dietary challenges, i.e. HF/high cholesterol *v.* HF, used. As in the present study, DIO mice were not challenged by dietary cholesterol, a potential cholesterol-lowering effect of ASTX may not have been initiated.

Fibrosis and necroinflammation are key events in the development of NASH, and hepatic stellate cells and macrophages play a major role in the pathogenesis⁽⁴⁾. Upon activation by oxidative stress or fibrogenic cytokines, hepatic stellate cells transform into myofibroblasts and produce extracellular matrix proteins⁽³⁷⁾. TGF β 1 is a cytokine that facilitates liver fibrosis⁽³⁸⁾ and hepatocyte apoptosis⁽³⁹⁾ and stimulates hepatic stellate cells to produce α -smooth muscle actin and procollagen type I, α 1, major markers of fibrosis⁽⁴⁰⁾. Therefore, the effect of ASTX supplementation on the expression of fibrogenic genes was determined. Although the mRNA expression of *TGF β 1* was drastically elevated, that of α -smooth muscle actin and procollagen type I, α 1 was not significantly altered in the liver of ASTX-supplemented mice. It is known that TGF β 1 is secreted as a latent form, which requires to be cleaved by proteases such as matrix metalloproteinase-2 and matrix metalloproteinase-9 and to be activated by reactive oxygen species^(41,42). Therefore, the absence of α -smooth muscle actin and procollagen type I, α 1 induction despite an increase in *TGF β 1* expression induced by ASTX supplementation indicates that the activation of TGF β 1 may be perturbed in ASTX-supplemented mice. The expression of *CD68* and *F4/80*, macrophage markers, was not significantly altered by ASTX supplementation, suggesting that it may not have an impact on hepatic inflammation in DIO mice. However, we observed that primary splenocytes isolated from ASTX-supplemented mice exhibited a decrease in the mRNA expression of *IL-6* upon LPS challenge compared with those isolated from control mice. Splenocytes consist of a variety

of immune cells, including T and B lymphocytes, dendritic cells, and monocytes, and IL-6 is mainly secreted by T cells and monocytes⁽⁴³⁾. Given that LPS stimulates primarily monocyte/macrophages, the present results indicate that ASTX supplementation may decrease the sensitivity of splenic monocytes to inflammatory stimuli.

The antioxidant effect of ASTX may play a critical role in the aforementioned beneficial effects of ASTX in DIO mice. In our previous study in *apoE*^{-/-} mice, ASTX was found to attenuate oxidative stress in the liver by increasing the expression of *NRF-2* and its target antioxidant genes⁽¹³⁾. In the present study also, we found a 2-fold increase in the mRNA expression of *NRF-2* and an increased expression of its target endogenous antioxidant genes in the liver of ASTX-supplemented mice when compared with that in control mice. NRF-2 plays a critical role in cellular defence against oxidative stress by inducing the transcription of antioxidant genes⁽⁴⁴⁾. Under basal conditions, NRF-2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1), a negative regulator of NRF-2. When cells are under oxidative stress, oxidative modifications or phosphorylations in Keap1 release NRF-2 and therefore NRF-2 enters the nucleus to activate the transcription of genes containing an antioxidant response element in their promoters⁽⁴⁵⁾. Therefore, given that ASTX is known to be a potent antioxidant^(10–12), the increase in the expression of *NRF-2* and its target genes is counterintuitive. Our observations that ASTX supplementation increased the expression of carnitine palmitoyltransferase-1 α and *ACOX-1* in *apoE*^{-/-} mice and *ACOX-1* in DIO mice led us to speculate that ASTX may activate PPAR α because the genes involved in fatty acid oxidation are under the transcriptional regulation of PPAR α in the liver. In support of this speculation, Jia *et al.*⁽⁴⁶⁾ demonstrated that ASTX is a PPAR α agonist. Therefore, the activation of PPAR α by ASTX could burden the mitochondrial machinery of oxidative phosphorylation due to elevated fatty acid β -oxidation, resulting in the production of reactive oxygen species. In this case, antioxidant mechanisms, possibly via the NRF-2 pathway, need to be turned on to offset the production of reactive oxygen species for the protection against oxidative damage. Further studies are required to investigate this possibility.

In conclusion, we demonstrated that 0.03% ASTX supplementation lowered plasma TAG concentrations, induced hepatic endogenous antioxidant gene expression, and attenuated inflammatory response of splenocytes in DIO mice. The effects of ASTX indicate that it may be able to prevent the development of NASH. However, as DIO mice in the present study did not develop NASH features, we were not able to determine whether ASTX supplementation could prevent obesity-associated NASH. Another mouse study is currently underway in our laboratory, in which mice are being exposed to a severe dietary challenge for a longer period of time than in the present study to induce NASH features. Nonetheless, the present study opens the possibility that ASTX could be used for preventing obesity-associated metabolic dysfunctions and inflammation.

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None of the authors has any conflicts of interest to declare.

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