

Lycopene and Apo-10'-lycopenoic Acid Have Differential Mechanisms of Protection against Hepatic Steatosis in β -Carotene-9',10'-oxygenase Knockout Male Mice^{1–3}

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Abstract

Background: Nonalcoholic fatty liver disease is positively associated with obesity and cardiovascular disease risk. Apo-10'-lycopenoic acid (APO10LA), a potential oxidation product of apo-10'-lycopenal that is generated endogenously by β -carotene-9',10'-oxygenase (BCO2) cleavage of lycopene, inhibited hepatic steatosis in BCO2-expressing mice.

Objective: The present study evaluated lycopene and APO10LA effects on hepatic steatosis in mice without BCO2 expression.

Methods: Male and female BCO2-knockout (BCO2-KO) mice were fed a high saturated fat diet (HSFD) with or without APO10LA (10 mg/kg diet) or lycopene (100 mg/kg diet) for 12 wk.

Results: Lycopene or APO10LA supplementation reduced hepatic steatosis incidence (78% and 72%, respectively) and severity in BCO2-KO male mice. Female mice did not develop steatosis, had greater hepatic total cholesterol (3.06 vs. 2.31 mg/g tissue) and cholesteryl ester (1.58 vs. 0.86 mg/g tissue), but had lower plasma triglyceride (TG) (229 vs. 282 mg/dL) and cholesterol (97.1 vs. 119 mg/dL) than male mice. APO10LA-mitigated steatosis in males was associated with reduced hepatic total cholesterol (18%) and activated sirtuin 1 signaling, which resulted in reduced fatty acids (FAs) and TG synthesis markers [stearoyl-coenzyme A (CoA) desaturase protein, 71%; acetyl-CoA carboxylase phosphorylation, 79%; AMP-activated protein kinase phosphorylation, 67%], and elevated cholesterol efflux genes (cytochrome P450 family 7A1, 65%; ATP-binding cassette transporter G5/8, 11%). These APO10LA-mediated effects were not mimicked by lycopene supplementation. Intriguingly, steatosis inhibition by lycopene induced peroxisome proliferator-activated receptor (PPAR) α - and PPAR γ -related genes in mesenteric adipose tissue (MAT) that increases mitochondrial uncoupling [cell death-inducing DNA fragmentation factor, α subunit-like effector a, 55%; PR domain-containing 16, 47%; uncoupling protein 3 (*Ucp3*), 55%], FA β -oxidation (PPAR α , 53%; very long chain acyl-CoA dehydrogenase, 38%), and uptake (FA transport protein 4, 29%; lipoprotein lipase 43%). Expressions of 10 MAT PPAR-related genes were inversely correlated with steatosis score, suggesting that lycopene reduced steatosis by increasing MAT FA utilization.

Conclusions: Our data suggest that lycopene and APO10LA inhibit HSFD-induced steatosis in BCO2-KO male mice through differential mechanisms. Sex disparity of BCO2-KO mice was observed in the outcomes of HSFD-induced liver steatosis and plasma lipids. *J Nutr* 2015;145:268–76.

Keywords: nonalcoholic fatty liver disease, hepatic cholesterol, lipid metabolism, sirtuin 1, obesity, plasma lipids, mitochondrial uncoupling, PPAR, carotenoid metabolism, mesenteric adipose tissue

Introduction

Nonalcoholic fatty liver disease (NAFLD)⁸ is a chronic liver disease that is observed in 75–100% of overweight and obese

adults and children (1). NAFLD describes a series of related disorders that can progress in stages from simple steatosis to fibrosis and cirrhosis (2). Patients with NAFLD are often associated with metabolic syndrome including insulin resistance and hypertriglyceridemia, as well as with increased cardiovascular disease (CVD) and related risk factors (3), which include

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³ Supplemental Figures 1–4 and Supplemental Tables 1–4 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

an atherogenic lipid profile (high in serum TGs and LDL cholesterol; low in HDL cholesterol) and systemic inflammation (4, 5). Liver has the capacity to synthesize lipids via de novo lipogenesis and secrete lipoprotein particles. Thus, liver dysfunction can modify risk factors of CVD (3, 4). In light of the current obesity epidemic and increasing prevalence of NAFLD, the prevention of NAFLD-associated metabolic disorders through dietary means represents an important strategy.

Observational data indicates that the intake of lycopene-rich foods is inversely associated with CVD risk (6, 7) and with CVD risk indicators such as dyslipidemia and systemic inflammation (6, 7). In vivo and in vitro studies have suggested that lycopene may use multifaceted mechanisms to reduce CVD and NAFLD risks (8–10), mechanisms that include modulating cholesterol metabolism, serving as an antioxidant, and inhibiting inflammation (8–10). Of note, NAFLD patients have substantially reduced plasma lycopene concentration (11), suggesting potential interactions between low lycopene status and CVD risk.

We and others have demonstrated that the enzyme β -carotene-9',10'-oxygenase (BCO2) plays a critical role in the endogenous metabolism of nonprovitamin A carotenoids including lycopene (12, 13). BCO2 metabolizes lycopene through asymmetric cleavage at the 9',10' double bond and generates metabolites, which include apo-10'-lycopenal, apo-10'-lycopenol, and apo-10'-lycopenoic acid (APO10LA; chemical structure shown in Supplemental Figure 1) (12, 13). Evidence suggests that these metabolites may exhibit more important biological roles than lycopene itself (9, 14, 15), and BCO2 ablation in mice altered lycopene metabolism (16, 17). In particular, APO10LA supplementation was effective in inhibiting hepatic steatosis in genetically induced obese (*ob/ob*) mice (15) and attenuating high saturated fat diet (HSFD)-induced liver inflammation as well as tumor number and volume in *C57Bl/6J* mice (18). An important question remains as to whether the biological activity of lycopene could be different from its metabolite APO10LA in the absence of BCO2 expression. This information is critically needed because 19 single nucleotide polymorphisms of BCO2 have been found in humans (19), and BCO2 gene variants can account for the differential expression and activity of the BCO2 enzyme among individuals. BCO2 single nucleotide polymorphisms have been associated with increased circulatory proinflammatory IL-18 expression (19) and with decreased HDL cholesterol concentrations (19), suggesting a gene-diet interaction between the BCO2 enzyme and dietary lycopene on human health outcomes. We hypothesize that the lycopene biological effects would be different from APO10LA in mice when BCO2 enzyme expression is absent.

⁸ Abbreviations used: Abc, ATP-binding cassette transporter; Acad, acyl-CoA dehydrogenase; ACC, acetyl-CoA carboxylase; *Acox1*, acyl-CoA oxidase 1; AMPK, AMP-activated protein kinase; APO10LA, apo-10'-lycopenoic acid; BCO2, β -carotene-9',10'-oxygenase; BCO2-KO, β -carotene-9',10'-oxygenase-knockout; BW, body weight; CE, cholesteryl ester; Cidea, cell death-inducing DNA fragmentation factor, α subunit-like effector a; CVD, cardiovascular disease; Cyp, cytochrome P450 family; Cysc, cytochrome c; Dgat, diglyceride acyltransferase; Fatp4, FA transport protein 4; FC, free cholesterol; FoxO, Foxhead box protein O; HO, heme-oxygenase; HSFD, high saturated fat diet; HSFD+APO10LA, high saturated fat diet supplemented with apo-10'-lycopenoic acid; HSFD+Ly, high saturated fat diet supplemented with lycopene; HSFD+LyP, high saturated fat diet supplemented with lycopene placebo beadlet; Hsl, hormone-sensitive lipase; MAT, mesenteric adipose tissue; MglI, monoacylglycerol lipase; NAFLD, nonalcoholic fatty liver disease; Pck, phosphoenolpyruvate carboxykinase; Pgc1 α , PPAR γ coactivator 1 α ; Pnpla2, patatin-like phospholipase domain containing 2; Prdm16, PR domain-containing 16; SCD1, stearoyl-CoA desaturase 1; SIRT1, sirtuin 1; Slc2a4, solute carrier family 2 type 4; TC, total cholesterol; Ucp3, uncoupling protein 3.

Using a murine model that lacks BCO2 protein expression, we investigated the separate effects of lycopene or APO10LA supplementation on HSFD-induced hepatic steatosis and determined the underlying mechanisms thereof.

Methods

Animal experimental design. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee at the Jean Mayer-USA Human Nutrition Research Center on Aging at Tufts University. BCO2-knockout (BCO2-KO) mice with BCO2 protein ablation were generated as previously described (20). The schematic for the animal experimental design is shown in Supplemental Figure 2. Mice were fed the standard laboratory chow until the start of experiments (7012 Teklad LM-485; Harlan Laboratories), maintained on a 12-h light/dark cycle in a controlled temperature and humidity room, and consumed water ad libitum. Two separate experiments were conducted concurrently to evaluate APO10LA and lycopene effects. Six-wk-old male and female BCO2-KO mice were randomly assigned to 1 of the 4 diets: 1) an obesogenic HSFD [Bio-Serv; information cited in Ip et al. (21)], in which 60% of energy is lard derived; 2) an HSFD supplemented with APO10LA (HSFD+APO10LA; 10 mg/kg diet); 3) an HSFD supplemented with lycopene placebo beadlet (HSFD+LyP; 1000 mg beadlet/kg diet); and 4) an HSFD supplemented with lycopene (HSFD+Ly; 100 mg lycopene/kg diet) for 12 wk. We included both male and female mice in our experiments because there are no previously published studies to our knowledge that examined the biological effects of APO10LA in BCO2-KO mice. All mice in the experiments were given fresh diets every 2–3 d and maintained on their respective diets until the experiment was completed. Mice were weighed weekly and killed at 18 wk of age by exsanguinations under deep isoflurane (Isothesia; Butler Schein) anesthesia without food deprivation.

Dietary supplementation. The APO10LA used in the experiments of this study was provided by Dr. Hansgeorg Ernst (BASF, Ludwigshafen, Germany) with 99% purity, analyzed by HPLC as previously described (Supplemental Figure 3) (13, 14). The lycopene supplement was in the form of a 10% lycopene beadlet (BASF). The composition of the lycopene placebo beadlet is listed in Supplemental Table 1. APO10LA, lycopene, or placebo beadlet without lycopene was incorporated directly into the HSFD to achieve a homogenous diet mixture and stored as previously described (18). We selected the same APO10LA dosage (10 mg/kg diet) as in our published study (18), where APO10LA supplementation for 24 wk reduced HSFD-promoted hepatic inflammation in *C57Bl/6J* male mice. This APO10LA-supplemented dose (10 mg/kg diet) is approximately equivalent to 0.36 mg/d APO10LA in a 60-kg adult man (22, 23). A/J mice supplemented with the same APO10LA dose for 14 wk had a plasma APO10LA concentration of \sim 1.0 nmol/L (14). This plasma concentration is comparable to the sum of apo-lycopenals (1.9 nmol/L) found in human plasma of individuals who had consumed tomato juice (with 21.8 mg/d lycopene) for 8 wk (24). Based on the assumption that BCO2-KO mice have similar carotenoid absorption as other strains of mice on a vitamin-supplemented, semipurified diet (\sim 1/10 of human absorption) (14, 15, 25), our lycopene-supplemented dose (100 mg/kg diet) is approximately equivalent to 8.1 mg/d lycopene in a 60-kg adult man (22, 23). The mean human dietary lycopene was \sim 8 mg/d (26, 27). In addition, our recently published study (28) showed that BCO2-KO mice supplemented with the identical lycopene dose for 24 wk had mean hepatic lycopene concentrations that were within human ranges (0.1–20.7 nmol/g tissue) (29).

Liver and adipose tissue collection and processing. After the mice were killed, whole livers and mesenteric adipose tissue (MAT) were removed from experimental mice and processed for biochemical and histologic analyses as described previously (18).

Histopathologic evaluation. Five- μ m sections of formalin-fixed, paraffin-embedded liver left lobes were processed and stained with

hematoxylin and eosin. Two independent investigators unaware of treatment groups evaluated the liver sections under light microscopy for steatosis magnitude and inflammation severity, as described previously (18), in 20 random fields at 100× magnification per sample. Briefly, the degree of steatosis was graded 0–4 (grading 0 = <5%, 1 = 5–25%, 2 = 26–50%, 3 = 51–75%, 4 = >75%), based on the mean percentage of fat-accumulated hepatocytes per field. Hepatic steatosis incidence was evaluated by the percentage of mice in each group with a steatosis grading >0. Inflammatory foci were evaluated by the number of inflammatory-cell clusters, which mainly constitute mononuclear inflammatory cells as described previously (18). Hepatic inflammatory foci incidence was evaluated by the percentage of mice in each group with ≥1 liver inflammatory foci.

Plasma lipid and lipoprotein profile. Blood from cardiac puncture was collected into EDTA-coated tubes, and plasma was separated from RBCs by centrifugation at 1500 × g for 20 min at 4°C. Plasma total cholesterol (TC), HDL cholesterol, and TG concentrations were determined on a Cobas Mira automated analyzer with use of enzymatic reagents (Roche Diagnostics). Non-HDL cholesterol was calculated as the difference between TC and HDL cholesterol.

Hepatic cholesterol and TG contents. Hepatic TC and free cholesterol (FC) contents of extracted liver lipids were analyzed by GC as described previously (30). Cholesteryl ester (CE) was computed as the difference between TC and FC (30). Liver tissue (25 mg of wet weight) was used to determine hepatic TG content as previously described (15). Hepatic cholesterol and TG were corrected for the wet liver weight used in the analysis.

RNA extraction and qRT-PCR. Total RNA extraction from frozen liver and MAT tissue and subsequent qRT-PCR were performed as described previously (18). Primer sequences are listed in **Supplemental Table 2**.

Protein isolation and Western blotting. Protein isolation from frozen liver tissue and subsequent Western blotting analysis of specific proteins were executed as described previously (18). The following antibodies

were used for western blotting: acetyl-CoA carboxylase (ACC), AMP-activated protein kinase (AMPK) α, phosphorylated-ACC (Ser79), phosphorylated-AMPKα (Thr172), stearoyl-CoA desaturase (SCD1) (Cell Signaling), IL-6 (R&D), acetylated-Foxhead box protein O1 (FoxO1), heme-oxygenase (HO)-1, and sirtuin 1 (SIRT1). Horseradish peroxidase-conjugated secondary antibody (Bio-Rad) was used to detect specific proteins, and the specific bands were visualized by a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce) according to the manufacturer's instructions. Dilution series and calibration curve were performed for each of the antibodies used to quantify protein. β-Actin protein was used for loading normalization of some proteins unless specified otherwise, which was detected by an anti-actin antibody (Sigma-Aldrich). A GS-710 Calibrated Imaging Densitometer (Bio-Rad) was used to quantify the intensities of protein bands.

Statistical analysis. SAS 9.3 software was used to perform the statistical analysis. Values in the text are means ± SEMs or medians (IQRs). Two-factor ANOVA was used to examine the overall, diet, sex, or diet × sex interaction effects on experimental outcomes modified by APO10LA or lycopene supplementation. Student's two-sample unequal variance *t* test, Wilcoxon signed-rank test, and χ-square test were used to examine the differences for the following comparisons: 1) HSF and HSF+APO10LA, and 2) HSF+LyP and HSF+Ly. Spearman's rank correlation was used to perform nonparametric measures of statistical dependence between 2 variables. The *P* value was set at 0.05 for the comparisons to reach statistical significance.

Results

APO10LA and lycopene supplementation ameliorated HSF-induced hepatic steatosis in BCO2-KO male mice. Final mean body and liver weights did not differ significantly between supplemented and nonsupplemented BCO2-KO mice (**Tables 1 and 2**). There was a marked difference in the response of female and male mice to the HSF for liver steatosis, cholesterol accumulation, and plasma lipid concentrations.

TABLE 1 Primary outcomes of BCO2-KO mice fed an HSF with or without APO10LA supplementation for 12 wk¹

	Experimental groups				Two-factor ANOVA			
	Males		Females		Overall	Diet × sex	Diet	Sex
	HSFD	HSFD+APO10LA	HSFD	HSFD+APO10LA				
Mice, <i>n</i>	10	12	10	12				
Liver weight, g	1.02 ± 0.03	1.02 ± 0.03	0.92 ± 0.04	0.97 ± 0.02	0.01	0.92	0.8	<0.01
Initial BW, g	19.6 ± 0.55	19.5 ± 1.46	16.3 ± 0.50	16.2 ± 0.31	<0.01	0.95	0.80	<0.01
Final BW, g	36.9 ± 1.62	36.3 ± 0.93	26.7 ± 1.37	27.4 ± 0.94	<0.01	0.59	0.99	<0.01
BW gain, g	17.3 ± 1.39	16.8 ± 0.87	10.3 ± 1.34	11.2 ± 0.73	<0.01	0.52	0.91	<0.01
Hepatic steatosis incidence, %	90	25*	0	0	NA	NA	0.09	<0.01
Hepatic steatosis score, median (range)	1 (0–2)	0 (0–2)*	0 (0–0)	0 (0–0)	NA	NA	0.06	<0.01
Hepatic inflammatory foci incidence, % of mice	40	0*	20	16	NA	NA	0.04	0.88
Hepatic lipids, mg/g wet tissue								
TC	2.46 ± 0.16	2.01 ± 0.09*	3.50 ± 0.37	2.69 ± 0.16*	<0.01	0.4	0.01	<0.01
FC	1.50 ± 0.05	1.36 ± 0.03*	1.51 ± 0.05	1.41 ± 0.04*	0.04	0.64	0.01	0.47
CE	0.96 ± 0.14	0.65 ± 0.08*	1.98 ± 0.34	1.28 ± 0.15*	<0.01	0.31	0.01	<0.01
TG	33.4 ± 1.89	29.2 ± 1.09 [†]	38.7 ± 5.09	33.4 ± 3.09	0.20	0.86	0.13	0.13
Plasma lipids								
TG, mg/dL	312 ± 22.1	252 ± 20.1	235 ± 19.0	222 ± 9.70	<0.01	0.19	0.04	<0.01
TC, mg/dL	117 ± 3.50	121 ± 5.21	97.4 ± 6.42	96.7 ± 4.67	<0.01	0.64	0.83	<0.01
HDL-C:TC, %	45.8 ± 0.89	47.4 ± 0.81	46.3 ± 0.98	47.2 ± 0.42	0.43	0.67	0.11	0.84
Non-HDL-C, mg/dL	63.7 ± 2.66	63.8 ± 2.89	52.7 ± 4.13	51.1 ± 2.60	<0.01	0.78	0.74	<0.01

¹Values are means ± SEMs or *n* (%) unless otherwise indicated. Two-factor ANOVA was used to examine the overall, diet, sex, and diet × sex effects. Symbols indicate different between HSF and HSF+APO10LA from HSF+LyP within sex: **P* < 0.05, [†]*P* = 0.07. APO10LA, apo-10'-lycopenic acid; BCO2-KO, β-carotene-9',10'-oxygenase-knockout; BW, body weight; CE, cholesteryl ester; FC, free cholesterol; HDL-C, HDL cholesterol; HSF, high saturated fat diet; HSF+APO10LA, high saturated fat diet supplemented with apo-10'-lycopenic acid; NA, not applicable; TC, total cholesterol.

TABLE 2 Primary outcomes of BCO2-KO mice fed an HSFd with or without lycopene supplementation for 12 wk¹

	Experimental groups				Two-factor ANOVA			
	Males		Females		Overall	Diet × sex	Diet	Sex
	HSFD+LyP	HSFD+Ly	HSFD+LyP	HSFD+Ly				
Mice, <i>n</i>	10	12	10	12				
Liver weight, g	1.04 ± 0.04	0.98 ± 0.03	0.89 ± 0.03	0.90 ± 0.02	<0.01	0.26	0.37	<0.01
Initial BW, g	19.5 ± 0.50	19.4 ± 0.40	16.2 ± 0.37	16.1 ± 0.28	<0.01	0.99	0.75	<0.01
Final BW, g	35.3 ± 1.27	34.5 ± 1.25	25.3 ± 1.13	26.7 ± 1.13	<0.01	0.36	0.9	<0.01
BW gain, g	15.8 ± 1.17	15.0 ± 1.34	9.04 ± 0.99	10.6 ± 0.92	<0.01	0.31	0.79	<0.01
Hepatic steatosis incidence, %	80	18*	0	0	NA	NA	0.02	<0.01
Hepatic steatosis score, median (range)	1 (0–2)	0 (0–2)*	0 (0–0)	0 (0–0)	NA	NA	0.02	<0.01
Hepatic inflammatory foci incidence, % of mice	30	8	0	8	NA	NA	0.53	0.14
Hepatic lipids, mg/g wet tissue								
TC	2.47 ± 0.30	2.28 ± 0.20	3.01 ± 0.21	3.04 ± 0.17	0.04	0.62	0.73	0.01
FC	1.50 ± 0.05	1.43 ± 0.04	1.50 ± 0.03	1.49 ± 0.04	0.44	0.38	0.25	0.45
CE	0.96 ± 0.27	0.85 ± 0.14	1.51 ± 0.19	1.55 ± 0.14	0.02	0.7	0.87	<0.01
TG	29.9 ± 2.32	24.2 ± 2.27 [†]	33.9 ± 2.44	30.0 ± 1.38	0.02	0.67	0.03	0.06
Plasma lipids								
TG, mg/dL	255 ± 17.1	249 ± 18.0	251 ± 18.7	234 ± 22.7	0.88	0.79	0.55	0.62
TC, mg/dL	109 ± 2.79	107.8 ± 4.04	96.5 ± 5.16	95.5 ± 5.79	0.08	0.96	0.79	0.01
HDL-C:TC, %	51.5 ± 1.11	46.8 ± 0.90	47.2 ± 0.76	47.5 ± 0.79	0.92	0.66	0.99	0.59
Non-HDL-C, mg/dL	57.9 ± 1.96	57.4 ± 2.51	51.2 ± 3.18	50.3 ± 3.56	0.15	0.94	0.79	0.02

¹Values are means ± SEMs or *n* (%) unless otherwise indicated. Two-factor ANOVA was used to examine the overall, diet, sex, and diet × sex effects. Symbols indicate different from HSFd+LyP within sex: **P* < 0.05, [†]*P* = 0.09. BCO2-KO, β-carotene-9',10'-oxygenase-knockout; BW, body weight; CE, cholesteryl ester; FC, free cholesterol; HDL-C, HDL cholesterol; HSFd, high saturated fat diet; HSFd+Ly, high saturated fat diet supplemented with lycopene; HSFd+LyP, high saturated fat diet supplemented with lycopene placebo; NA, not applicable; TC, total cholesterol.

Male mice weighed significantly more than female mice at the start and end of the experiments (Tables 1 and 2). The percentage weight gain was 63.4 (10.3 g) in the female mice and 83.3 (16.2 g) in the male mice. In male mice, APO10LA and lycopene supplementation significantly decreased HSFd-induced liver steatosis incidence and score (Figure 1, Tables 1 and 2). APO10LA or lycopene supplementation tended to decrease hepatic TGs (Tables 1 and 2). In female mice, the HSFd did not induce steatosis, and APO10LA or lycopene supplementation did not alter hepatic steatosis outcome or TGs (Tables 1 and 2). In both male and female mice, APO10LA, but not lycopene supplementation, significantly reduced plasma TG and hepatic FC and CE concentrations (Tables 1 and 2). Final body weight (BW), BW gain, and hepatic FC and TC were correlated with steatosis score in both female and male mice ($R^2 = 0.52, 0.52, 0.33, 0.35$; $P = 0.001, 0.001, 0.030, 0.020$, respectively). Female mice had significantly greater hepatic TC and CE than males, but FC was not significantly different between males and females (Tables 1 and 2). Female mice had less weight gain, and plasma TGs, TC, and non-HDL cholesterol than males (Tables 1 and 2). Female mice had lower plasma TGs than males only within the HSFd and HSFd+APO10LA groups (Table 1). These results suggest that lycopene and APO10LA had biological effects in the absence of BCO2. Sex disparity exists on diet-induced hepatic steatosis and lipid metabolism in BCO2-KO mice.

APO10LA, but not lycopene, decreased HSFd-induced hepatic inflammation in BCO2-KO male but not female mice. APO10LA, but not lycopene supplementation, significantly reduced hepatic inflammatory foci incidence in male but not female BCO2-KO mice (Table 1). This APO10LA-mediated inflammation suppression in male mice was associated with reduced hepatic IL-6 protein (94%, $P = 0.06$; Figure 2A) and with elevated anti-inflammatory HO-1 protein (74%; Figure 2B) expression in male mice.

APO10LA increased hepatic SIRT1 protein, deacetylation of FoxO1, and AMPKα phosphorylation in male mice. APO10LA, but not lycopene supplementation, significantly increased hepatic SIRT1 protein expression (58%; Figure 2C) and induced the deacetylation of SIRT1 direct target FoxO1 (33%; Figure 2D) in male mice compared to the HSFd mice. These observations were associated with a significant increase in AMPKα phosphorylation (Thr172, 67%; Figure 2E) and decreased SIRT1 protein cleavage (64%; Figure 2F). Hepatic SIRT1 protein cleavage was inversely correlated with SIRT1 protein ($R^2 = -0.50, P = 0.001$) and HO-1 protein ($R^2 = -0.59, P < 0.001$) in the HSFd and HSFd+APO10LA mice but not in the HSFd+Ly and HSFd+LyP mice. These data suggest that APO10LA mitigated HSFd-induced steatosis through activating SIRT1 signaling.

APO10LA-induced hepatic SIRT1 signaling was associated with decreased lipogenic biomarkers expression and with upregulated cholesterol efflux genes in male mice. Supplementation of APO10LA, but not lycopene, significantly reduced expression/activation of proteins involved in hepatic lipogenesis, which included ACC inhibition by phosphorylation (Ser79, 79%; Figure 2G), reduced SCD1 protein expression (71%; Figure 2H), and decreased *Scd1* gene expression (40%; Figure 3A). Hepatic *Scd1* gene expression was significantly correlated with SCD1 protein ($R^2 = 0.64, P = 0.003$), SIRT1 protein ($R^2 = -0.31, P = 0.044$), and hepatic steatosis score ($R^2 = 0.45, P = 0.002$) in HSFd and HSFd+APO10LA mice. Hepatic ACC phosphorylation was significantly correlated with SIRT1 protein expression ($R^2 = 0.43, P < 0.001$) in all mice. APO10LA also significantly increased hepatic cholesterol efflux gene ATP-binding cassette transporter (*Abcg5/8*) expression (11%; Figure 3A) and bile synthesis gene cytochrome P450 family (*Cyp7a1*) expression (65%; Figure 3A), and had no significant effect on

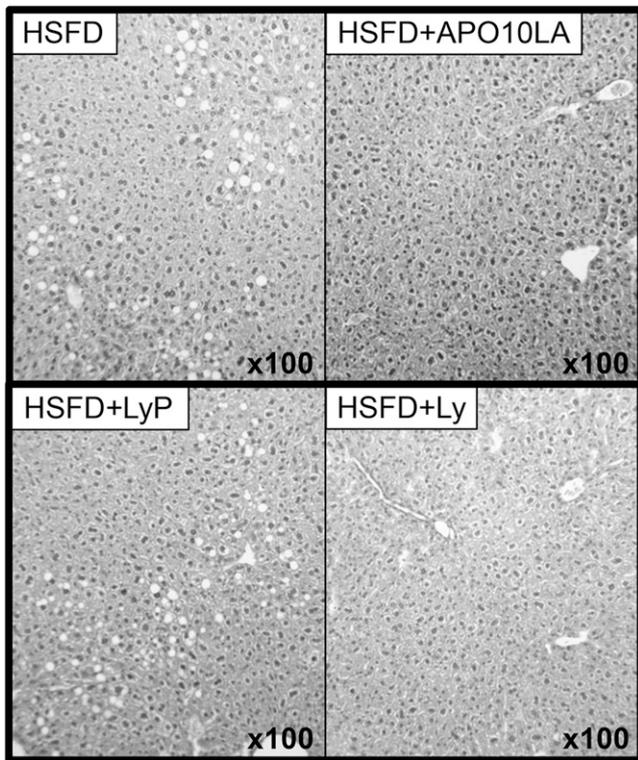


FIGURE 1 Histopathology of liver of BCO2-KO male mice that were or were not supplemented with lycopene or APO10LA for 12 wk. Representative hematoxylin-and-eosin-stained livers from HSF, HSF+APO10LA, HSF+LyP, and HSF+Ly mice. APO10LA, apo-10'-lycopenoic acid; BCO2-KO, β -carotene-9',10'-oxygenase-knockout; HSF, high saturated fat diet; HSF+APO10LA, high saturated fat diet supplemented with apo-10'-lycopenoic acid; HSF+Ly, high saturated fat diet supplemented with lycopene; HSF+LyP, high saturated fat diet supplemented with lycopene placebo beadlet.

Abcb11 or *Cyp8b1* gene expression (Figure 3A). In addition, hepatic SIRT1 protein expression was correlated with *Abcg5/8* gene expression in all mice ($R^2 = 0.32$, $P = 0.005$). APO10LA or lycopene supplementation did not alter other genes involved in hepatic cholesterol metabolism (*Abca1*, *Abcg1*, *ApoA1*, and *ApoB*; data not shown). Female mice had lower expression of the *Abca1*, *ApoA1*, and *ApoB* genes but higher expression of the *Abcg1* gene than males (Figure 3B). These data suggest that APO10LA reduced hepatic steatosis and cholesterol through inhibiting FA synthesis while promoting FA β -oxidation and cholesterol efflux. Female BCO2-KO mice exhibited differential hepatic cholesterol metabolism compared to male mice.

Lycopene upregulated PPAR α -inducible genes in MAT but not in hepatic tissue in male mice. PPAR α is a transcription factor involved in lipid metabolism including FA β -oxidation, FA uptake, and lipolysis (31). We observed that lycopene supplementation, but not APO10LA, in BCO2-KO mice significantly induced PPAR α genes (53%; Figure 3C) and PPAR α -inducible genes including very-long-chain acyl-CoA dehydrogenase (*Acad-VL*, 38%; Figure 3C), FA transport protein 4 (*Fatp4*, 29%; Figure 3C), and lipoprotein lipase (43%; Figure 3C) in BCO2-KO male mice. Similar trends were observed in other PPAR α -associated genes in MAT [including medium-chain *Acad* (*Acad-M*), acyl-CoA oxidase 1 (*Acox1*), *Cd36*, *Fatp1*, hormone-sensitive lipase (*Hsl*), monoacylglycerol lipase (*Mgll*), and patatin-like phospholipase domain containing 2 (*Pnpla2*); Figure 3C and Supplemental Figure 4]. In addition, PPAR α gene

expression in MAT was significantly correlated with a number of PPAR α -inducible genes (Supplemental Table 3). Lycopene-induced *Acad-M* and *Pnpla2* gene expression were inversely correlated with hepatic steatosis score ($R^2 = -0.31$, $P = 0.018$; $R^2 = -0.23$, $P = 0.070$, respectively). APO10LA or lycopene supplementation had no effects on hepatic PPAR α -associated genes (*Acad-M*, *Cd36*, *Cpt*, *Fatp2*, *Fatp5*, and PPAR α) in BCO2-KO male mice (data not shown). These data suggest that PPAR α -associated gene induction by lycopene was tissue specific in the absence of BCO2.

Lycopene-mitigated steatosis in male mice was associated with elevated expression of PPAR γ -associated genes in MAT. Activation of the transcription factor PPAR γ was shown to promote mitochondrial uncoupling and expression

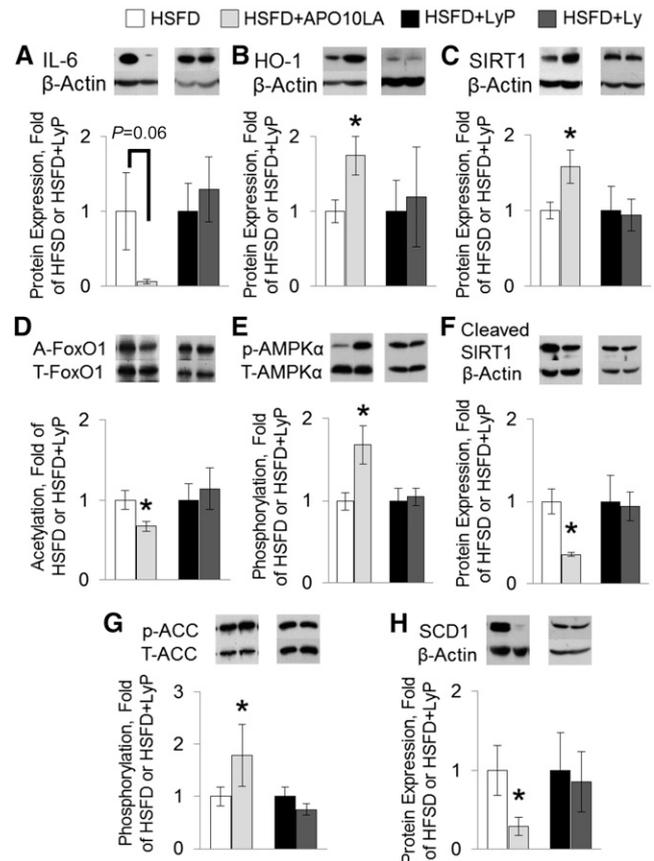


FIGURE 2 Hepatic molecular markers in BCO2-KO male mice that were or were not supplemented with lycopene or APO10LA for 12 wk. Protein expression in liver lysates (HSF, HSF+APO10LA, HSF+LyP, and HSF+Ly male BCO2-KO mice). β -Actin was used as loading control unless specified otherwise. Graphical representation of fold changes in (A) IL-6; (B) HO-1; (C) SIRT1; (D) FoxO1 acetylation (FoxO1 as loading control); (E) phosphorylated AMPK α (Thr172; AMPK α as loading control); (F) cleaved-SIRT1; (G) phosphorylated ACC (Ser79; ACC as loading control); and (H) SCD1. Representative western blots with 1 sample per group are shown. Fold changes normalized to HSF. Values are means \pm SEMs, $n = 10$ –12. *Different from HSF, $P < 0.05$. A-, acetylated; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; APO10LA, apo-10'-lycopenoic acid; BCO2-KO, β -carotene-9',10'-oxygenase-knockout; FoxO, Foxhead box protein O; HO, heme-oxygenase; HSF, high saturated fat diet; HSF+APO10LA, high saturated fat diet supplemented with apo-10'-lycopenoic acid; HSF+Ly, high saturated fat diet supplemented with lycopene; HSF+LyP, high saturated fat diet supplemented with lycopene placebo beadlet; p-, phosphorylated; SCD1, stearoyl-CoA desaturase 1; SIRT1, sirtuin 1.

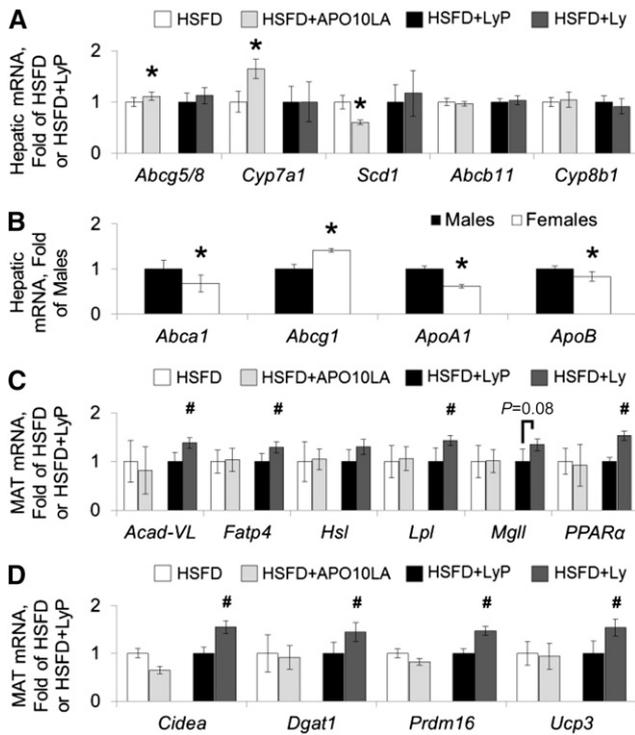


FIGURE 3 Hepatic and mesenteric adipose mRNA expression in BCO2-KO male mice that were or were not supplemented with lycopene or APO10LA for 12 wk. mRNA expression in liver or mesenteric adipose tissue lysates [(A, C, D) HSFd, HSFd+APO10LA, HSFd+LyP, and HSFd+Ly male BCO2-KO mice, $n = 10$ –12/group; (B) males or females, $n = 42$ /group]. Graphical representation of fold changes in (A) hepatic mRNA of *Abcg5/8*, *Cyp7a1*, *Scd1*, *Abcb11*, *Cyp8b1*; (B) hepatic mRNA of *Abca1*, *Abcg1*, *ApoA1*, *ApoB*; (C) PPAR α -associated genes: *Acad-VL*, *Fatp4*, *Hsl*, *Lpl*, *MglI*, *PPAR α* ; and (D) PPAR γ -associated genes: *Dgat1*, *Cidea*, *Prdm16*, *Ucp3*. Fold changes normalized to HSFd, HSFd+LyP, or males. Values are means \pm SEMs. *Different from HSFd or males, #Different from HSFd+LyP, $P < 0.05$. *Abc*, ATP-binding cassette transporter; *Acad*, acyl-CoA dehydrogenase; APO10LA, apo-10'-lycopenoic acid; BCO2-KO, β -carotene-9',10'-oxygenase-knockout; *Cidea*, cell death-inducing DNA fragmentation factor, α subunit-like effector a; *Cyp*, cytochrome P450 family; *Dgat1*, diglyceride acyltransferase; *Fatp*, FA transport protein; HSFd, high saturated fat diet; HSFd+APO10LA, high saturated fat diet supplemented with apo-10'-lycopenoic acid; HSFd+Ly, high saturated fat diet supplemented with lycopene; HSFd+LyP, high saturated fat diet supplemented with lycopene placebo beadlet; *Hsl*, hormone-sensitive lipase; MAT, mesenteric adipose tissue; *MglI*, monoacylglycerol lipase; *Prdm16*, PR domain containing 16; *Scd1*, stearoyl-CoA desaturase 1; *Ucp3*, uncoupling protein 3.

of FA utilizing genes (31). Lycopene, but not APO10LA, supplementation significantly increased MAT expression of PPAR γ -associated genes including cell death-inducing DNA fragmentation factor, α subunit-like effector a (*Cidea*, 55%; Figure 3D), diglyceride acyltransferase 1 (*Dgat1*, 45%; Figure 3D), PR domain-containing 16 (*Prdm16*, 47%; Figure 3D), and uncoupling protein 3 (*Ucp3*, 55%; Figure 3D) in BCO2-KO male mice. Similar trends were observed in other PPAR γ -associated genes [including *Cd36*, cytochrome c (*Cytc*), phosphoenolpyruvate carboxykinase (*Pck1*), PPAR γ , PPAR γ coactivator 1 α (*Pgc1 α*), solute carrier family 2 type 4 (*Slc2a4*), and *Ucp1*; Figure 3D and Supplemental Figure 4] in MAT. Lycopene-induced *Cytc*, *Dgat1*, *Pgc1 α* , PPAR γ , *Prdm16*, *Slc2a4*, *Ucp1*, and *Ucp3* gene expression were inversely correlated with hepatic steatosis score ($R^2 = -0.46$, -0.24 , -0.48 , -0.42 , -0.55 , -0.35 , -0.26 , and -0.55 , respectively; $P = 0.001$, 0.056 , 0.007 , 0.001 , 0.001 , 0.011 , 0.029 , and

0.035 , respectively). PPAR γ gene and PPAR γ -associated genes were significantly correlated with each other (Supplemental Table 4). These data suggest that lycopene induced MAT PPAR γ -associated genes in the absence of BCO2 and inhibited steatosis by upregulating MAT FA utilization.

Discussion

The present study evaluated the effects of lycopene and APO10LA supplementation on hepatic steatosis in male and female mice that were deficient in BCO2 expression. BCO2-KO male mice developed hepatic steatosis as examined by histology after 12 wk of being fed an HSFd, suggesting that this in vivo model can be useful in evaluating dietary effects on liver steatosis. Supplementation with either lycopene or APO10LA inhibited high-saturated-fat-induced hepatic steatosis incidence and severity in BCO2-KO male mice. Dietary lycopene supplementation mitigated steatosis despite the absence of BCO2 enzyme expression, suggesting that lycopene itself exhibits biological functions. It should be noted that APO10LA or lycopene supplementation tended to decrease hepatic TGs in BCO2-KO male mice, but the effects were not significant ($P = 0.07$ and 0.09 , respectively). This discrepancy between our histopathologic steatosis grading and hepatic TGs was likely because of the regional distribution of steatosis in the liver and the small sample size that led to high variability within each experimental group. Therefore, we believe that the histopathologic steatosis grading of left liver lobes was more valid in evaluating hepatic steatosis.

In the present study, the biochemical and molecular analyses of liver and MAT suggest that lycopene and APO10LA have differential mechanisms of biological action in the BCO2-KO mice. APO10LA mitigated steatosis and inflammation in BCO2-KO male mice coincided with induced SIRT1 protein, deacetylation of SIRT1 direct downstream target, and FoxO1, as well as with reduced SIRT1 protein cleavage. These results suggest that APO10LA modulated SIRT1 protein in a post-translational manner, which is similar to our previous findings (18). APO10LA-mediated steatosis reduction and SIRT1-signaling activation in BCO2-KO mice were associated with decreased lipogenic SCD1 gene and protein as well as with increased AMPK activation and ACC phosphorylation. Importantly, *Scd1* gene expression in APO10LA-supplemented BCO2-KO mice correlated with hepatic steatosis score and negatively correlated with SIRT1 protein. SIRT1-signaling activation protected mice from HSFd-induced steatosis and inflammation through inhibiting FA biosynthesis (32, 33), promoting FA β -oxidation (32, 33), inhibiting *Scd* gene induction (34), and activating AMPK (35). AMPK is an enzyme that inhibits ACC by phosphorylation to the inactive form of the enzyme (36). Hepatic ACC activation promotes FA biosynthesis and steatosis while inhibiting FA β -oxidation (34–36). Therefore, these data suggest that APO10LA reduced steatosis through SIRT1-signaling activation. Moreover, the APO10LA biological effects in the present study are in agreement with our previous findings using mouse strains with BCO2 expression (15, 18), indicating that APO10LA may not be further metabolized by the BCO2 enzyme despite the presence of a 9',10' double bond in its structure.

Hepatic FC accumulation was shown to sensitize rats to cytokine-mediated steatohepatitis (37). In the present study, the decreased hepatic FC in APO10LA-fed BCO2-KO male mice coincided with reduced hepatic inflammation and IL-6 expression ($P = 0.06$) as well as elevated HO-1 expression. APO10LA also induced hepatic cholesterol efflux and bile synthesis genes, without

altering other genes involved in hepatic cholesterol metabolism. APO10LA supplementation stimulated HO-1 in the lungs of A/J mice (14). The SIRT1-activator resveratrol induced hepatic HO-1 (38), cholesterol efflux, and bile synthesis genes (39). In contrast, hepatocyte-specific deletion of SIRT1 in mice suppressed hepatic *Cyp7a1* and promoted hepatic cholesterol accumulation, steatosis, and inflammation (33). Notably, APO10LA-induced hepatic *Abcg5/8* in the present study was correlated with SIRT1 protein. *Abcg5/8* and *Cyp7a1* are also targets of the transcription factor farnesoid X receptor (40), but APO10LA did not modulate 2 other farnesoid X receptor-modifiable genes, *Abcb11* and *Cyp8b1*. Therefore, these results suggest that APO10LA modulated *Abcg5/8* and *Cyp7a1* genes through SIRT1 activation.

In the present study, lycopene protected BCO2-KO male mice against HSF-induced hepatic steatosis despite the absence of hepatic SIRT1-signaling modulations. Lycopene can accumulate in adipose tissue (10) and modulate adipose lipid/inflammation-associated signaling cascades (41, 42). Evidence showed that the portal vein directly connects the liver with the visceral MAT (43, 44), and visceral fat content is highly correlated with the degree of liver steatosis in humans (45, 46). In the present study, the hepatic steatosis reduction by lycopene was associated and inversely correlated with induced PPAR α - and PPAR γ -associated genes in MAT. PPAR α activation can result in the net effect of reducing FA synthesis and secretion from cells (47). PPAR γ activation in adipose tissue increases adipocyte differentiation, FA sequestration, and FA utilization (48, 49). Therefore, lycopene-mediated upregulation in MAT PPAR α - and PPAR γ -associated genes may have promoted MAT FA utilization, and subsequently reduced lipid delivery from MAT to the liver. Nevertheless, it should be noted that lycopene effects on PPAR α - and PPAR γ -mediated signaling were relatively modest; thus, this might explain why lycopene did not reduce final BW. Lycopene modulations in PPAR-associated signaling were observed in the adrenal glands, liver, and kidney of rodents in previous studies (16, 50, 51). During the preparation of this manuscript, a recent publication by Tan et al. (50) showed that 3-wk-old BCO2-KO male mice on a lycopene-supplemented (250 mg/kg diet) AIN-93G semipurified diet for 3 wk modulated hepatic PPAR-associated signaling that could potentially affect hepatic responses to metabolic, infectious, or chemical stress. However, the present study showed that lycopene in HSF-fed BCO2-KO mice only modified PPAR α -associated genes in MAT and not in the liver. This discrepancy between the present study and the findings published by Tan et al. may be the result of the differential experimental designs, diet composition, and/or lycopene-supplemented dosage/duration. Future research is needed to examine whether BCO2 expression can modify tissue specificity of lycopene modulations in PPAR α -associated signaling.

The sex difference in BW gain and hepatic steatosis outcomes have previously been demonstrated in a number of mouse and rat models (52–55). Indeed, the present study showed that HSF feeding induced hepatic steatosis in male but not in female BCO2-KO mice. BCO2-KO females gained less BW and exhibited differential hepatic lipid metabolism compared to males. NAFLD patients are more likely to be males and postmenopausal women (56), and the difference in NAFLD prevalence may be attributed to the putative protective effects of estrogen. Estrogen treatment inhibited hepatic lipid storage in estrogen-deficient mice (57, 58) and decreased BW in HSF-fed *C57Bl/6J* female mice (59). Therefore, the sex disparity on HSF-induced steatosis and BW in the present study may be the difference in circulatory estrogen between male and female BCO2-KO mice. Other factors including the elevated *Abcg1* gene in BCO2-KO females compared to males may also protect females from hepatic lipid accumulation. This is

because targeted disruption of *Abcg1* in mice resulted in accumulation of neutral lipids and phospholipid in hepatocytes (60).

Female BCO2-KO mice in the present study had greater hepatic TC and CE and lower plasma TGs, TC, and non-HDL cholesterol but no differences in HDL cholesterol compared to male mice. These observations in BCO2-KO females were associated with lower *Abca1*, *ApoA1*, and *ApoB* gene expression, but greater *Abcg1* gene expression than male mice. ApoB is required for the assembly and secretion of intestinal and hepatic apolipoproteins that transport CE and TG (61). Therapeutic inhibition of ApoB synthesis reduced plasma TC and TG in humans (61). Therefore, it is plausible that the reduced ApoB expression in BCO2-KO females compared to males may contribute to the sex disparity in plasma lipids. Hepatic cholesterol efflux to HDL requires ABCG1, whereas efflux to the HDL precursor ApoA1 requires ABCA1 (60). Because female BCO2-KO mice had elevated *Abcg1* but reduced *Abca1* and *ApoA1* genes compared to males, these results may explain why male and female BCO2-KO mice had similar HDL cholesterol.

Because we aimed to isolate the differential effects of lycopene and APO10LA by using mice without BCO2 expression, we did not include wild-type mice in the present study. However, we acknowledge that the scope of the present study would be broadened with the inclusion of wild-type mice. In addition, BCO2 is believed to be the key enzyme involved in lycopene eccentric metabolism (12, 13), and BCO2-KO mice had elevated lycopene accumulation in tissue and serum compared to wild-type mice (16, 17). Lycopene-supplemented mice without carotene-15,15'-oxygenase (BCO1) expression did not have elevated lycopene accumulation in tissue and serum compared to wild-type mice (16, 17). BCO1 also did not cleave lycopene in vitro in previous studies (10, 62). However, recent studies suggested that lycopene could be metabolized to acyclo-retinal by purified recombinant human BCO1 (63) and by recombinant murine BCO1 (64). Further investigations with BCO1/BCO2-double KO mice are currently ongoing in our laboratory to examine if lycopene-mediated functions are the effects of lycopene metabolites generated by BCO1 cleavage.

Taken together, the results from the present study suggest that both lycopene and APO10LA supplementation were effective in reducing high saturated fat-induced liver steatosis in mice where BCO2 expression was absent. The APO10LA-mediated ability to prevent hepatic steatosis and inflammation in male mice was associated with SIRT1-signaling activation in the liver, whereas the lycopene-mediated ability to prevent hepatic steatosis was associated with modulations in MAT lipid metabolism. Female BCO2-KO mice were less prone to HSF-induced liver steatosis, weight gain, and elevated plasma TGs and TC than male mice.

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