Prevention and Reversal of Lipotoxicity-Induced Hepatic Insulin Resistance and Steatohepatitis in Mice by an Antioxidant Carotenoid, β-Cryptoxanthin

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Excessive hepatic lipid accumulation promotes macrophages/Kupffer cells activation, resulting in exacerbation of insulin resistance and progression of nonalcoholic steatohepatitis (NASH). However, few promising treatment modalities target lipotoxicity-mediated hepatic activation/polarization of macrophages for NASH. Recent epidemiological surveys showed that serum β-cryptoxanthin, an antioxidant carotenoid, was inversely associated with the risks of insulin resistance and liver dysfunction. In the present study, we first showed that β-cryptoxanthin administration ameliorated hepatic steatosis in high-fat diet-induced obese mice. Next, we investigated the preventative and therapeutic effects of β-cryptoxanthin using a lipotoxic model of NASH: mice fed a high-cholesterol and high-fat (CL) diet. After 12 weeks of CL diet feeding, β-cryptoxanthin administration attenuated insulin resistance and excessive hepatic lipid accumulation and peroxidation, with increases in M1-type macrophages/Kupffer cells and activated stellate cells, and fibrosis in CL diet-induced NASH. Comprehensive gene expression analysis showed that β-cryptoxanthin down-regulated macrophage activation signal-related genes significantly without affecting most lipid metabolism-related genes in the liver. Importantly, flow cytometry analysis revealed that, on a CL diet, β-cryptoxanthin caused a predominance of M2 over M1 macrophage populations in parallel, β-cryptoxanthin decreased lipopolysaccharide-induced M1 marker mRNA expression in peritoneal macrophages, whereas it augmented IL-4-induced M2 marker mRNA expression, in a dose-dependent manner. Moreover, β-cryptoxanthin reversed steatosis, inflammation, and fibrosis progression in preexisting NASH in mice. In conclusion, β-cryptoxanthin prevents and reverses insulin resistance and steatohepatitis, at least in part, through an M2-dominant shift in macrophages/Kupffer cells in a lipotoxic model of NASH. (Endocrinology 156: 987–999, 2015)
Nonalcoholic fatty liver disease (NAFLD) has emerged as a major public health problem worldwide. It has become increasingly evident that obesity or ectopic fat induces an innate immune response with subsequent recruitment of immune cells, such as macrophages and T cells, leading, ultimately, to the development of insulin resistance and nonalcoholic steatohepatitis (NASH). Thus, NAFLD can be defined as a lipotoxic liver injury and can impair whole-body insulin resistance as well as progress to NASH (1, 2). Previously, we developed a cholesterol- and saturated fatty acid-induced model of lipotoxic NASH and observed that excessive hepatic lipid accumulation promoted the activation of macrophages/Kupffer cells, resulting in the exacerbation of insulin resistance and hepatic inflammation and fibrogenesis (3). Importantly, hepatic inflammation, fibrosis, and hepatocellular ballooning, characteristic features of human NASH, developed after 12 weeks of feeding in this model.

Although insulin resistance and increased oxidative stress are believed to be the major causes of the progression to NASH, many agents including insulin sensitizers have been tested with disappointing results in the management of NASH; only vitamin E has yielded some promise in the treatment of patients with NASH. The lipophilic antioxidant vitamin E was associated with reduced hepatic steatosis and lobular inflammation (4). However, there is a continuing need for additional and more effective therapies for patients with NASH.

It is known that micronutrient antioxidants, such as vitamins and carotenoids, are decreased in both the serum and liver tissue of patients with chronic liver disease and cirrhosis (5–7). Recently, low serum concentrations of carotenoids, such as α-carotene and β-carotene, as well as vitamin E, were shown to be associated with obesity (7–9). Thus, these micronutrient antioxidant deficiencies may also contribute to the development of greater adiposity and comorbidities, such as insulin resistance and NASH. Importantly, carotenoids are as potent in inhibiting lipid peroxidation as vitamin E (10). However, carotenoids have not been fully tested as candidate NASH treatments. One such molecule may be the antioxidant carotenoid, β-cryptoxanthin.

β-Cryptoxanthin is a xanthophyll carotenoid that is relatively abundant in human plasma (11, 12). Serum β-cryptoxanthin concentrations have been found to be inversely associated with indices of oxidative DNA damage and lipid peroxidation (13). Recent epidemiological studies have also shown that high serum β-cryptoxanthin lowers the risk of insulin resistance and alcohol-induced increases in serum γ-glutamyltransferase in nondiabetic subjects (11, 12). Additionally, animal experiments and in vitro studies have shown that β-cryptoxanthin has anti-inflammatory effects, primarily by modulating the innate immune response induced by macrophages (14).

Thus, we hypothesized that the administration of β-cryptoxanthin would exert a beneficial effect on the development of NASH by suppressing oxidative stress, the innate immune response, and/or insulin resistance. We have recently found that β-cryptoxanthin ameliorates diet-induced NASH by suppressing inflammatory gene expression in mice (15). However, the mechanisms have not been fully elucidated. In addition, it is unknown whether β-cryptoxanthin could improve preexisting NASH. Thus, the preventative and therapeutic effects of β-cryptoxanthin and potential mechanisms were investigated using a lipotoxic model of NASH: mice fed a high-cholesterol and high-fat (HF) (CL) diet in this study. We determined that β-cryptoxanthin prevented and reversed insulin resistance and steatohepatitis by regulating both macrophage accumulation and M1/M2 status in a diet-induced lipotoxic model of NASH.

Materials and Methods

Mice and diets

Seven-week-old male C57BL/6J mice were purchased from Charles River Laboratories and used for experiments after 1 week of adaptation. For hepatic steatosis experiments, mice were fed normal chow (NC) diet with 10% of calories from fat (CRF-1; Charles River), HF diet with 60% of calories from fat (Research Diets, Inc), and with or without 0.003% β-cryptoxanthin for 10 weeks. For NASH experiments, mice were fed either NC or a CL (also referred to as atherogenic high fat; Research Diets, Inc) with 60% of calories from fat, 1.25% cholesterol, 0.5% sodium cholate (3), and with or without 0.003% β-cryptoxanthin for 12 weeks (n = 5 for NC and NC containing 0.003% β-cryptoxanthin [NC + CX]; n = 8 for HF and HF containing 0.003% β-cryptoxanthin; and n = 8 for CL and CL containing 0.003% β-cryptoxanthin [CL + CX]). All mice were maintained on a 12-hour light, 12-hour dark cycle and given free access to food and water.

All animal procedures were performed in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University.

Purification of β-cryptoxanthin

Nonesterified β-cryptoxanthin for experiments was prepared and processed as described (15, 16). Briefly, raw centrifuged pulp from Satsuma mandarin was subjected to enzymatic degradation, and then a precipitate was recovered after tubular centrifugation. β-Cryptoxanthin was extracted from the acetone-substituted precipitate with hexane. After hexane removal, the extract was sequentially separated into soluble and insoluble portions with hexane, acetone, ethanol, and a hexane/ethanol (3:7) mixture at −30°C. β-Cryptoxanthin was concentrated in the hexane-soluble, acetone-soluble, ethanol-insoluble, and hexane/ethanol-insoluble portions. The concentrated hexane/ethanol-insoluble portion was dissolved in hexane/ethanol (1:1)
and hydrolyzed with 10% potassium hydroxide in ethanol overnight at room temperature. After the addition of water, the organic phase was recovered and concentrated. The insoluble substance was filtered and recrystallized from ethanol to afford nonesterified β-cryptoxanthin by filtration. According to HPLC analysis, the purity of the β-cryptoxanthin obtained was 96%.

β-Cryptoxanthin concentration measurement

After 8-week-old C57BL/6J mice were fed a CL or CL diet with 0.003% of β-cryptoxanthin for 3 weeks and then fasted for 2 hours, tissues were collected and snap frozen in liquid nitrogen. β-Cryptoxanthin concentrations in tissues were measured by HPLC and reported as μg/g organ.

Biochemical analyses

Plasma triglycerides (TGs), total cholesterol (TC), nonesterified fatty acids (NEFA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, and insulin levels and hepatic TG, TC, NEFA, and hydroxyproline concentrations were measured as described previously (3, 17). Liver thiobarbituric acid reactive substrates (TBARSs) were extracted and measured according to the instructions with the Cayman Chemical TBARS assay kit.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

After 12 weeks of feeding, a GTT was conducted after an overnight fast. After baseline blood collection, mice were injected ip with glucose (2 g/kg). One week later, an ITT was performed after a 4-hour fast. Mice were injected ip with human insulin (0.5 U/kg). For insulin signaling in the liver, mice were injected iv with human insulin (5 U/kg) 1 week after ITT, and liver samples were snap frozen for further analysis.

Fat tolerances tests and TG secretion tests

For fat tolerance tests, 8-week-old male C57BL/6J mice were fasted for 16 hours, followed by oral gavage with a single dose of pure olive oil (20 μL/g body weight [BW]) with or without β-cryptoxanthin (60 mg/kg). Plasma TG levels were determined before and at 1, 2, 4, 6, and 8 hours after olive oil loading. For TG secretion tests, 8-week-old mice were administered olive oil with or without β-cryptoxanthin (2 mg/kg/d) for 2 weeks. Mice were then fasted for 16 hours, injected iv with 15% Triton WR1339 (5 μL/g BW), and blood samples were collected at baseline and 30, 60, and 120 minutes after the Triton injection.

Histological examination and immunohistochemistry

Paraffin wax-embedded liver sections were stained with hematoxylin and eosin (H&E), Azan, Sirius Red, and immunohistochemically for EGF-like-module-containing mucin-like hormone receptor-like 1 (F4/80) or α-smooth muscle actin (α-SMA) as described previously (3, 17). Staining with Azan, Sirius Red, anti-α-SMA, and anti-F4/80 antibodies was examined morphometrically and quantified as a percentage of the field using the ImageJ and SPSS software, as described previously (3, 17).

Quantitative real-time PCR (qPCR)

Total RNA was isolated from frozen liver using a GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qPCR was performed on a CFX384 (Bio-Rad) using the SYBR Green Master Mix, as described previously (17). Primers used for real-time PCR are shown in Supplemental Table 1.

Immunoblots

Tissues were homogenized and sonicated in radio-immunoprecipitation assay lysis buffer (Millipore), supplemented with protease and phosphatase inhibitors (Roche Diagnostics). The primary antibodies used were anti-phospho-c-Jun N-terminal kinase (JNK) (Thr183/Tyr185) (9255), anti-JNK (9258), anti-phospho-p38 MAPKs (Thr180/Tyr182) (9211), anti-p38 MAPK (9212), anti-phospho-nuclear factor κ light chain-enhancer of activated B cells (NF-κB) p65 (Ser536) (3033), anti-NF-κB (3034), anti-phospho-insulin receptor β-subunit (IRB) (Tyr1146) (3021), anti-IRβ (3025), anti-phospho-Ser473 Protein kinase B (Akt) (9271), anti-Akt (9272) (Cell Signaling Technology, Inc), anti-α-SMA (sc-53141; Santa Cruz Biotechnology, Inc), and monoclonal anti-β-actin (A5441) (Sigma-Aldrich).

Fluorescence-activated cell sorting (FACS) analysis

The left lobes of livers were lysed gently and digested for 20 minutes at 37°C with type IV collagenase (Sigma-Aldrich) and type I deoxyribonuclease in PBS containing 2% BSA (pH 7.4). Nonparenchymal cells were incubated with Fc-Block (BD Bioscience), followed by incubation with fluochrome-conjugated antibodies (Supplemental Table 2). Cells were analyzed using a FACSAria II (BD Bioscience) as described previously (17). Data analysis and compensation were performed using FlowJo (Tree Star).

DNA microarray analysis

Comprehensive gene expression analysis was previously performed using a GeneChip Mouse Genome 430 2.0 array (Affymetrix) (15). The data have been deposited in the NCBI Gene Expression Omnibus (18) and are accessible through Gene Expression Omnibus Series accession number GSE51432 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51432). In this study, we reanalyzed the data using GeneSpring version 11.5 (Agilent Technologies) and Ingenuity Pathway Analysis (Ingenuity Systems), and focused on the β-cryptoxanthin-improved gene sets associated with “physiological system development and functions.”

Isolation of peritoneal macrophages and primary hepatocytes

Eight-week-old male C57BL/6J mice were injected with 4% thioglycolate and 48 hours after peritoneal macrophages were isolated and cultured in DMEM (Gibco) with 10% fetal bovine serum. After starved for 6 hours, cells were coincubated with lipopolysaccharide (LPS) (1 μg/mL) or IL-4 (10 ng/mL) and β-cryptoxanthin (50μM–200μM) for 24 hours.

Mouse primary hepatocytes were isolated from 8-week-old male C57BL/6J mice as described (19). After culturing in DMEM without fetal bovine serum for 6 hours, cells were treated with...
400μM oleic acid (OA) (Sigma-Aldrich) and β-cryptoxanthin (0μM, 50μM, 100μM, and 200μM) for 24 hours before harvested. To measure lipid content in the hepatocytes, cells were fixed in 10% formalin for 1 hour, washed with 60% isopropanol, and stained with Oil Red-O (Sigma-Aldrich) solution for 10 minutes. The cells were then repeatedly washed with water, photographed, and destained in 100% isopropanol for 15 minutes. The optical density of the isopropanol solution was measured at 500 nm. Cellular TG levels were measured as described (20).

Statistical analyses
All data are presented as means ± SEM. Differences in mean values between 2 groups were assessed using a two-tailed Student’s t-test. Differences in mean values among more than 2 groups were determined by ANOVA. P < .05 was considered to indicate statistical significance.

Results
β-Cryptoxanthin reduced hepatic steatosis and decreased lipid accumulation in hepatocytes
To determine the effect of β-cryptoxanthin on hepatic steatosis, β-cryptoxanthin was administered to HF diet-induced obese mice. After 10 weeks of feeding, β-cryptoxanthin administration significantly reduced hepatic steatosis and TG accumulation in HF diet-induced obese mice, without affecting weight and adiposity (Figure 1, A–C). To further assess the effects of β-cryptoxanthin on lipid accumulation in hepatocytes, we incubated primary hepatocytes with OA with or without β-cryptoxanthin. β-Cryptoxanthin treatment decreased lipid accumulation in a dose-dependent manner, as assessed by Oil Red-O staining and cellular TG content in lipid-loaded primary hepatocytes (Figure 1D).

To confirm the role of β-cryptoxanthin in lipolysis, we performed a fat tolerance test to assess the contri-
bution of β-cryptoxanthin in the clearance of exogenously infused TG. After dietary TG absorption, plasma TG levels increased, peaking after 4 hours (Figure 1E). In β-cryptoxanthin-treated mice, the plasma TG levels began to decrease 2 hours after olive oil infusion, and this effect was maintained until 6 hours, suggesting enhanced plasma TG lipolysis due to β-cryptoxanthin. However, liver TG secretion was not significantly affected by β-cryptoxanthin (Figure 1F). Together, these results suggest that β-cryptoxanthin inhibited lipid accumulation in the hepatocytes and activated lipolysis, contributing to the amelioration of hepatic steatosis.

**β-Cryptoxanthin ameliorated diet-induced NASH in a dose-dependent manner**

Because β-cryptoxanthin markedly improved the fatty liver condition in HF diet-induced mice, we next determined whether it prevented the development of NASH. To determine the most effective doses of β-cryptoxanthin on NASH, C57BL/6J mice were fed the CL, or CL diet containing 0.001% or 0.003% β-cryptoxanthin for 12 weeks. After feeding with the 0.003% β-cryptoxanthin-containing CL diet, accumulation of β-cryptoxanthin was seen in serum and various tissues (Figure 2A). The β-cryptoxanthin concentration in the liver was highest among the tissues examined. Treatment with β-cryptoxanthin dose dependently ameliorated liver pathology and decreased plasma AST and ALT (Figure 2, B and C) in diet-induced NASH. The effect was more prominent in the 0.003% β-cryptoxanthin treated group, so we performed further investigations in that group (named CL + CX).

**β-Cryptoxanthin improved dyslipidemia and liver dysfunction in a lipotoxic NASH model**

After 12 weeks of feeding, β-cryptoxanthin significantly decreased plasma TG, NEFA, AST, and ALT in CL mice, whereas it decreased plasma TG and NEFA in NC mice. Body weight, food intake, liver and epididymal fat weight, and plasma TC were unaffected by β-cryptoxanthin in NC and CL mice (Table 1). These results indicated that β-cryptoxanthin improved dyslipidemia and liver dysfunction in NASH mice.

**β-Cryptoxanthin prevented the development of hepatic steatosis by suppressing lipogenic gene expression**

Consistent with our previous study (15), NC, NC+CX, CL, and CL+CX mice had similar body weights (Supple-
mental Figure 1A) and consumed similar quantities of food (Table 1). However, histological analysis revealed severe lipid accumulation in the liver of CL group, accompanied with increased hepatic TG, TC, and NEFA levels, which were decreased markedly by β-cryptoxanthin (Supplemental Figure 1, B and C). TBARS levels in the liver were increased by CL diet feeding, revealing exaggerated lipid peroxidation in livers of NASH mice. β-Cryptoxanthin treatment resulted in less hepatic lipid peroxidation (Supplemental Figure 1C).

During the development of steatohepatitis, expression of lipogenic regulator genes, including sterol regulatory element-binding transcription factor 1c, liver X receptor α, carbohydrate-responsive element-binding protein, peroxisome proliferator-activated receptor γ coactivator 1 α and β, fat-specific protein, and fatty acid synthesis genes, including fatty acid synthase and stearoyl-coenzyme A desaturase 1, were increased significantly in the liver of CL vs NC mice (Supplemental Figure 1D). Treatment with β-cryptoxanthin suppressed the expression of these lipogenic genes. However, no significant difference was seen in the expression of mitochondrial fatty acid β-oxidation genes between CL and CL+CX mice (Supplemental Figure 1E). Together, these results suggest that β-cryptoxanthin reduced lipid accumulation in the liver of CL+CX mice by suppressing lipogenic gene expression.

**β-Cryptoxanthin alleviated diet-induced impaired glucose homeostasis and insulin resistance**

GTTs indicated that administration of β-cryptoxanthin had no effect on glucose tolerance in NC-fed mice (Supplemental Figure 2). However, CL diet-induced glucose intolerance, insulin resistance and hyperinsulinemia in both the fasting and fed states were suppressed significantly by β-cryptoxanthin (Figure 3, A–C). Next, we evaluated insulin signaling in the livers of the mice (Figure 3D). Insulin-stimulated Tyr phosphorylation of IRβ, and Ser phosphorylation of Akt were enhanced in the liver of CL+CX mice vs CL mice. Thus, the preventative effect on steatohepatitis due to β-cryptoxanthin administration was associated with protection from diet-induced hepatic insulin resistance and glucose intolerance.

**β-Cryptoxanthin reduced the activation of both Kupffer cells and stellate cells and attenuated hepatic inflammation and fibrosis**

The CL diet causes hepatic steatosis and insulin resistance but also induces intense inflammation in the liver (3). β-Cryptoxanthin markedly reduced the number of F4/80+ cells in the livers of CL mice, as assessed by immunostaining and mRNA expression as described previously (Figure 4A and Supplemental Figure 3, A and B) (15). Our results further demonstrated that gene expression of proinflammatory cytokines, including TNFα, IL-1β, and IL-6, was up-regulated by CL diet feeding, whereas β-cryptoxanthin decreased the expression of these genes significantly (Figure 4A). These findings were also associated with attenuation of the phosphorylation of JNK, p38 MAPK, and NF-κB p65 (Figure 4B). Thus, β-cryptoxanthin reduced the infiltration and activation of Kupffer cells, resulting in the attenuation of hepatic inflammation in NASH mice.

Histological analyses with Azan and Sirius Red staining revealed that CL diet alone induced fibrosis, as described previously (3). β-Cryptoxanthin prevented the development of hepatic fibrosis (Supplemental Figure 3, A and B). Importantly, β-cryptoxanthin lowered the hydroxyproline contents, a biochemical marker of hepatic collagen content, to normal levels (Figure 4C). The CL diet-induced increase in α-SMA-positive cells was also decreased by β-cryptoxanthin (Supplemental Figure 3, A and B), and

<table>
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<tr>
<th>Parameters at 12 Weeks of Treatment</th>
<th>NC</th>
<th>NC+CX</th>
<th>CL</th>
<th>CL+CX</th>
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</thead>
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<tr>
<td>Body weight (g)</td>
<td>31.6 ± 1.1</td>
<td>30.9 ± 0.5</td>
<td>33.2 ± 0.8</td>
<td>32.3 ± 0.7</td>
</tr>
<tr>
<td>Food intake (g/d/kg BW)</td>
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<td>86.3 ± 2.7</td>
<td>94.9 ± 3.4</td>
<td>99.6 ± 4.4</td>
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<tr>
<td>Liver weight (g)</td>
<td>1.10 ± 0.06</td>
<td>1.15 ± 0.03</td>
<td>1.61 ± 0.05b</td>
<td>1.59 ± 0.04b</td>
</tr>
<tr>
<td>Epididymal fat weight (g)</td>
<td>0.60 ± 0.08</td>
<td>0.53 ± 0.09</td>
<td>0.90 ± 0.12b</td>
<td>0.84 ± 0.11</td>
</tr>
<tr>
<td>Plasma TG (mg/dL)</td>
<td>92.9 ± 4.7</td>
<td>69.7 ± 5.6a</td>
<td>60.2 ± 2.4b</td>
<td>46.7 ± 7.3bc</td>
</tr>
<tr>
<td>Plasma TC (mg/dL)</td>
<td>91.1 ± 3.4</td>
<td>85.9 ± 7.4</td>
<td>168.3 ± 3.2b</td>
<td>153.9 ± 14.7b</td>
</tr>
<tr>
<td>Plasma NEFA (mEq/L)</td>
<td>1.22 ± 0.08</td>
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<td>1.08 ± 0.10b</td>
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<td>Plasma AST (IU/L)</td>
<td>13.2 ± 2.2</td>
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<td>40.0 ± 1.7b</td>
<td>30.1 ± 1.5bcd</td>
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<td>Plasma ALT (IU/L)</td>
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<td>19.7 ± 1.5b</td>
<td>11.0 ± 1.9bcd</td>
</tr>
</tbody>
</table>

Data were obtained from 20-week-old fasted mice on different diets. Data are presented as means ± SEM (n = 5 for NC and NC+CX groups and n = 8 for CL and CL+CX groups).

* a P < 0.05, vs mice fed NC diet.

* b P < 0.01, vs mice fed NC diet.

* c P < 0.05, vs mice fed the CL diet.

* d P < 0.01, vs mice fed the CL diet.
this was further confirmed by immunoblotting and qPCR (Figure 4, C and D). Additionally, the CL diet up-regulated mRNA expression of TGFβ1, collagen type I α1 and plasminogen activator inhibitor-1, vs the NC diet, whereas β-cryptoxanthin administration decreased expression of these fibrogenic genes (Figure 4D). Together, these results indicate that β-cryptoxanthin decreased collagen accumulation by inhibiting the activation of hepatic stellate cells (HSCs) in the liver, leading to attenuation of hepatic fibrosis.

**Coordinated down-regulation of both macrophage activation and T-cell differentiation-related genes in the NASH liver by β-cryptoxanthin**

Comprehensive gene expression analysis using a DNA microarray showed the gene sets that were significantly up- or down-regulated by CL diet or β-cryptoxanthin (Supplemental Figure 4A). Ingenuity Pathway Analysis showed that β-cryptoxanthin most significantly suppressed the genes associated with “hematological system development and function” in the physiological system development and functions among 507 analyzed genes (Supplemental Table 3). Notably, β-cryptoxanthin significantly decreased the expression of genes related to “activation of macrophages” and “quantity of T lymphocytes” (Table 2 and Supplemental Figure 4B). The genes associated with “antigen presentation pathway,” which is related to macrophages and T-cell differentiation, were significantly up-regulated by the CL diet and down-regulated by β-cryptoxanthin (Supplemental Figure 4C). Thus, β-cryptoxanthin suppressed inflammation and the resulting fibrosis, likely primarily by suppressing the increase in, and activation of, macrophages and T cells.
Reciprocal decrease in M1-type macrophages and an increase in M2-type macrophages in the livers of β-cryptoxanthin-fed mice

A reduction in hepatic macrophage activation and improved insulin action due to β-cryptoxanthin administration prompted us to investigate the macrophage subsets in the liver. To quantify hepatic M1- and M2-type macrophages in mice, we performed FACS analysis (Supplemental Figure 5). Consistent with the results of the immunohistological examinations, the total number of hepatic macrophages increased in mice fed the CL diet by 1.8-fold compared with NC-fed mice (Supplemental Figure 6). However, CL/CX mice had a slightly decreased total macrophage content compared with CL mice (Figure 5, A and B). On the NC diet, no difference was observed in either cluster of differentiation (CD)11c+CD206− (M1-type) or CD11c−CD206+ (M2-type) expression within macrophages from NC and NC/CX mice (data not shown). However, on a CL diet, in addition to a reduction in total hepatic macrophage content, CL/CX mice had 47% fewer M1-type macrophages and 87% more M2-type macrophages than CL mice, resulting in a predominance of the M2 over the M1 macrophage population (Figure 5B). However, a predominance of a lymphocyte antigen 6C (Ly6C) over Ly6Ch monocyte population was not observed in either peripheral blood or the bone marrow of CL/CX mice (Supplemental Figure 7). This suggests that β-cryptoxanthin causes a dynamic shift to an M2-dominant macrophage phenotype within the liver of NASH mice.

The hepatic total CD3+ T-cell content was also lower in CL+CX mice than CL mice (Figure 5C). Indeed, the total numbers of CD3+, CD4+, and CD8+ T cells were decreased by 31%, 38%, and 47%, respectively (all P < .05), in livers of the CL+CX group (Supplemental Figure 6). Thus, β-cryptoxanthin suppressed the accumulation of helper and cytotoxic T cells. In parallel, β-cryptoxanthin (50 μM–200 μM) decreased LPS-induced M1 marker mRNA expression (TNFα, IL-1β, and chemokine [C-C motif] ligand 5) in peritoneal macrophages, whereas it augmented IL-4-induced M2 marker mRNA expression (IL-10, CD209a, and mannose receptor C type 2) in a dose-dependent manner (Figure 5E and F). Thus, β-cryptoxanthin improved hepatic insulin resistance and inflam-
mation thorough an M2-dominant shift in macrophages/Kupffer cells and a subsequent reduction in T-cell accumulation in NASH.

β-Cryptoxanthin reversed advanced NASH in mice

Although we demonstrated that β-cryptoxanthin prevented the development of NASH markedly, another important question was whether β-cryptoxanthin could improve preexisting NASH. To that end, we examined the therapeutic effects of β-cryptoxanthin on advanced-stage NASH in the model mice. After NASH was developed by feeding CL diet for 12 weeks, the CL diet with or without β-cryptoxanthin was administered for an additional 12 weeks (Figure 6A). β-Cryptoxanthin treatment decreased plasma TG, NEFA, AST, and ALT of CL mice significantly, although body and liver weight and plasma TC levels were similar (Supplemental Table 4). β-Cryptoxanthin also decreased plasma insulin levels, improved glucose tolerance and enhanced hepatic insulin signaling in CL mice (Supplemental Table 4 and Supplemental Figure 8, A and B). Histologically, β-cryptoxanthin ameliorated macrovascular steatosis markedly, macrophage/Kupffer cell infiltration and fibrosis associated with HSCs activation (Figure 6B). Both hepatic lipid accumulation and peroxidation were suppressed significantly by β-cryptoxanthin (Figure 6C). Furthermore, β-cryptoxanthin inhibited intense liver fibrosis and hepatic inflammation through decreased activation of HSCs and attenuated inflammatory signaling (Figure 6D and Supplemental Figure 8, C and D). Taken together, these findings suggest that β-cryptoxanthin reversed the progression of NASH in mice.

Discussion

A link between lipotoxicity and the development of insulin resistance and its metabolic consequences, including NAFLD/NASH, is becoming clearer. However, it remains unclear whether strategies targeting the lipotoxicity-mediated activation of immune cells are useful for the prevention or treatment of NASH.

Our study revealed that β-cryptoxanthin prevented the development of NASH and reversed preexisting NASH in mice. The mechanism involved β-cryptoxanthin inhibiting the progression of lipid accumulation and peroxidation in the NASH liver (Figure 6E). Furthermore, β-cryptoxanthin reduced the accumulation of T cells and macrophages and regulated the M1/M2 status of macrophages/Kupffer cells in the liver without affecting the recruitment of monocytes from bone marrow (Figure 6E). Although the reduction in F4/80+ macrophages shown by immunostaining in the liver of β-cryptoxanthin-treated mice is not consistent with the less significant decrease in macrophage content measured by flow cytometry, the inconsistency may be due to differences in the antibodies used.

Tissue macrophages are phenotypically heterogeneous and have been characterized according to their activation/polarization state as M1 or “classically activated” proinflammatory macrophages or M2 or “alternatively activated” noninflammatory macrophages (21, 22). Dysregulation of the M1/M2 phenotypic balance is emerging as a central mechanism underlying the pathogenesis of chronic inflammatory disease (21). More recently, favorable properties of M2-polarized macrophages have been found in terms of a protective effect...
against insulin resistance (22), atherosclerosis (23), alcoholic liver disease, and NAFLD (24). Recently, Wan et al reported that M2-type Kupffer cells were suggested to protect against NAFLD by inducing M1-type Kupffer cell apoptosis (24). Thus, strategies restraining M1 polarization and/or driving alternative M2 activation of macro-

Figure 5. Decreased M1-type and increased M2-type macrophages in the NASH liver due to β-cryptoxanthin administration. A and B, A representative plot and quantitation of total macrophages and M1/M2 macrophages in the livers of mice. C and D, A representative plot of CD3⁺ T cells and quantitation of CD3⁺, CD8⁺, CD4⁺ T cells in liver. n = 8; *, P < .05, vs CL group. E and F, Macrophage marker mRNA expressions in peritoneal macrophages (n = 6). *, P < .01, vs control incubations; #, P < .05; ##, P < .01, vs LPS- or IL-4-stimulated conditions.
phages/Kupffer cells may protect against exacerbated inflammation and thus halt NASH progression.

Comprehensive gene expression analysis demonstrated that although β-cryptoxanthin reduced steatosis histologically, it was more effective in inhibiting inflammatory gene expression in NASH (15). In this study, we found that β-cryptoxanthin reduced the changes in genes associated with cell death, inflammatory responses, infiltration, and the activation of macrophages and other immune cells, such as T cells, and free-radical scavenging. Furthermore, pathway analysis demonstrated that the expression profile of macrophages, CD4+ helper T cells, and CD8+ cytotoxic T cells was induced significantly in NASH but decreased by β-cryptoxanthin (Table 2 and Supplemental Figure 4C).

Hepatic inflammation in NASH is associated with increased accumulation of not only M1-polarized macrophages but also T cells, in response to oxidative stress. Sutti et al showed that lipid peroxidation-induced liver recruitment of CD4+ and CD8+ T cells, in turn, further stimulated a macrophage M1 response in the methionine-choline-deficient diet model of NASH (25). Our result showing that β-cryptoxanthin decreased the accumulation of both CD4+ and CD8+ T cells suggests that β-cryptoxanthin suppressed recruitment of T cells as well as M1 activation of macrophages in the liver.

In mice, Ly6C+ monocytes accumulate in atherosclerotic plaques and show a proinflammatory response (26), whereas Ly6C− monocytes participate in the resolution of inflammation (27). Given the link between the monocyte subtypes and their fate as M1/M2 macrophages in NASH, β-cryptoxanthin did not affect Ly6C+ or Ly6C− monocyte subsets at the level of either the bone marrow or peripheral blood. Addi-
tionally, β-cryptoxanthin directly augmented IL-4-induced M2 or the alternative activation of macrophages in vitro (Figure 5F). These data indicate that β-cryptoxanthin caused a dynamic shift in the M2 polarization of macrophages/Kupffer cells within the NASH liver.

It is important that β-cryptoxanthin reduced hepatic lipid accumulation and peroxidation without affecting total body weight, fat weight, or food intake. This observation suggests that improvements in hepatic steatosis and hypertriglyceridemia due to β-cryptoxanthin were not secondary to a reduction in caloric intake, weight, or adiposity. Oxidative stress affects lipid accumulation and peroxidation, causing insulin resistance, inflammation, and fibrosis through activation of Kupffer cells and HSCs in the liver (2, 3). Our data suggest that β-cryptoxanthin, predominantly accumulated in the liver, could reduce the increased oxidative stress, as shown by the TBARS results, inhibiting lipid peroxidation and subsequent development of insulin resistance, inflammation, and fibrosis in the liver.

Hepatic steatosis and increased secretion of TG-rich lipoproteins, particularly very low-density lipoprotein, are characteristic features of insulin resistance, metabolic syndrome, and type 2 diabetes (20). To address the question regarding the fate of the accumulated lipids in the liver and the role of β-cryptoxanthin in their relocation, we determined its effects on hepatic lipid metabolism. First, we found that β-cryptoxanthin decreased liver TG significantly, probably through the suppression of lipogenic genes, such as sterol regulatory element-binding transcription factor 1c and its downstream genes. Second, β-cryptoxanthin did not alter hepatic TG secretion but enhanced TG clearance in response to exogenously infused TG (Figure 1, E and F), suggesting enhanced lipolysis by β-cryptoxanthin. Lowered plasma TG by β-cryptoxanthin may also be caused by differences in intestinal absorption or other factors; therefore, further studies are required to clarify the mechanism by which β-cryptoxanthin affects lipid metabolism. We suggest that β-cryptoxanthin decreased hypertriglyceridemia by promoting plasma TG lipolysis.

Carotenoids and tocopherols are major natural micronutrients that protect against free radical-mediated liver damage (5, 6). Importantly, β-cryptoxanthin is readily absorbed, and in a Japanese population, its serum concentration was highest among the 6 major carotenoids detectable in humans: α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, and zeaxanthin (11, 12). In rodents, the bioavailability of β-cryptoxanthin is higher than that of β-carotene (28); in accordance with that study, the serum β-cryptoxanthin concentration increased markedly to 35.5 ± 3.9 ng/mL in our NASH model (Figure 2A). The concentration of β-cryptoxanthin used in this study (~0.003%) was much lower than the concentration of vitamin E used in an animal study by NASH (29). Thus, our results showing that β-cryptoxanthin reversed advanced NASH indicate the potential of β-cryptoxanthin as a promising additional or more-effective treatment modality for NASH than vitamin E. In addition, β-cryptoxanthin is a provitamin A carotenoid that can be converted to retinoid. Besides its general antioxidant effect, β-cryptoxanthin may act through the retinoid receptor (30) or other nuclear receptors to show greater effectiveness on NASH.

In summary, we provide evidence that β-cryptoxanthin, an antioxidant carotenoid, prevented and reversed insulin resistance and steatohepatitis in a lipotoxic model of NAFLD by suppressing excessive lipid accumulation and peroxidation. The beneficial effects of β-cryptoxanthin were due to both a decrease in the hepatic recruitment of immune cells, such as T cells and macrophages, and an M2-dominant shift in macrophages/Kupffer cells. Thus, β-cryptoxanthin may be a promising treatment for NASH. These data also indicate that hepatic M2 polarization or alternative activation of macrophages/Kupffer cells can contribute to the attenuation of lipid-induced insulin resistance and hepatic inflammation and fibrogenesis.

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