

Molecular mechanisms of xanthophyll β -cryptoxanthin actions on high-refined carbohydrate diet-induced fatty liver disease and hepatocellular carcinoma in mice

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most commonly occurring chronic liver disease worldwide. NAFLD is one of the major risk factors for hepatocellular carcinoma (HCC), a primary liver cancer. Recent epidemiological studies have suggested that high consumption of a high-refined carbohydrate diet (HRCD) containing excessive amounts of refined sugar is associated with the development of NAFLD and metabolic syndrome-associated HCC. We previously showed that replacing a high-fat diet with a HRCD significantly exacerbates NAFLD and induces comparable HCC development in mice. However, mechanisms underlying the association of HRCD with NAFLD and HCC have not yet been elucidated and need to be investigated. Given the epidemic of obesity and NAFLD as well as the increased incidence of HCC, prevention of HRCD-related inflammation and tumorigenesis by dietary means represents an important disease control strategy. β -Cryptoxanthin (BCX) is a pro-vitamin A carotenoid primarily found in sweet red peppers, and butternut squash. BCX is preferentially cleaved by β -carotene-15,15'-oxygenase (BCO1) and β -carotene-9',10'-oxygenase (BCO2) to produce vitamin A and apo-carotenoids. The BCO1/BCO2 polymorphisms have been associated with alterations in carotenoid level status, consequently impacting the generation of vitamin A and apo-carotenoids. Recent accumulating animal studies have suggested that BCO1 and BCO2 play a role beyond carotenoid cleavage activity, such as tumor suppressing function. However, it remains unknown whether BCX can prevent the development of HRCD-induced NAFLD and HCC, if the protective effect is due to the BCX itself or its metabolites, and whether BCO1 and BCO2 themselves play a role in NAFLD and HCC.

The overall objectives of this dissertation are to investigate: 1) the role of carotenoid cleavage enzymes (BCO1 and BCO2) other than carotenoid cleavage activity (**Project 1**); 2) the effects of dietary BCX on the development of HRCD-induced NAFLD (**Project 2**), and 3) the effects of dietary BCX on diethylnitrosamine (DEN)-initiated, HRCD-promoted HCC in mice (**Project 3**).

In Project 1, by using BCO1/BCO2 double knock out (DKO) mice and their corresponding wild-type (WT) littermates placed on a regular chow diet, we showed that BCO1/BCO2 DKO mice developed hepatic steatosis (8/8) and had significantly higher levels of hepatic and plasma triglyceride and total cholesterol compared to WT (0/8) at 30 weeks of age. Hepatic changes in the BCO1/BCO2 DKO mice were associated with the following: 1) increases in lipogenesis markers; 2) upregulation of the cholesterol synthesis marker; and 3) increases in the hepatic oxidative stress marker (HO-1). This project revealed that BCO1/BCO2 could play a significant role in maintaining normal hepatic lipid and cholesterol homeostasis.

In Project 2, WT and BCO1/BCO2 DKO mice were randomly fed HRCD (66.5 % of energy from carbohydrate) with or without BCX (10 mg/kg diet) for 24 weeks. We found that BCX reduced hepatic steatosis severity and hepatic total cholesterol levels in both WT and DKO mice, compared to HRCD littermates ($P < 0.01$). Hepatic concentrations of BCX, but not retinol and retinyl palmitate, were 33-fold higher in DKO mice than in WT mice ($P < 0.001$). BCX feeding increased the hepatic fatty acid oxidation protein, PPAR α , and the cholesterol efflux gene, *Abcg5*, in WT but not DKO mice ($P < 0.05$). BCX consumption decreased the levels of hepatic lipogenesis proteins, ACC and SCD1 (3- and 5-fold, respectively), and those of the cholesterol synthesis genes, *Hmgcr* and *Hmgs1* (2.7- and 1.8-fold, respectively). In addition, BCX feeding increased the cholesterol catabolism gene *Cyp7a1* (1.9-fold) in the DKO but not WT mice ($P < 0.05$). BCX feeding increased hepatic protein levels of SIRT1 (2.5-fold) and AMPK (9-fold), and decreased the levels of hepatic FXR protein (80%) in DKO but not WT mice ($P < 0.05$). This project

demonstrated that BCX consumption mitigates HRCD-induced NAFLD in both WT and BCO1/BCO2 DKO mice through different mechanisms.

In Project 3, WT and DKO mice were injected with the hepatic carcinogen, DEN (25 mg/kg b.w.) at 2 weeks of age. At 6 weeks of age, all mice were fed either HRCD with or without BCX for 24 weeks. We observed that both WT and DKO fed BCX had significantly lower HCC multiplicity (58-60%), average tumor size (21-24%), and total tumor volume (51-58%), and hepatic steatosis scores, compared to the HRCD littermates. BCX feeding increased hepatic vitamin A levels in WT mice, but not in DKO mice that showed a significant accumulation of hepatic BCX. The chemopreventive effects of BCX were associated with increased levels of p53 protein acetylation and gluconeogenesis markers (PEPCK, G6Pase) and decreased protein levels of a glycolysis marker (LDHA) and hypoxia-inducible factor-1 α (HIF-1 α), as well as its downstream targets, matrix metalloproteinase 2/9 in tumors. However, we did not find significant differences in liver tumor outcomes between WT and BCO1/BCO2 DKO mice injected with DEN and fed HRCD alone. This last project demonstrated that BCX feeding alleviated HRCD-promoted HCC progression by modulating the hypoxic tumor microenvironment, glucose metabolism, and acetylation of p53 in the liver tumors of WT and BCO1/BCO2 DKO mice. Additionally, we provided the first *in vivo* evidence using BCO1/BCO2 ablated mice to demonstrate that BCO1 and BCO2 do not play a major role as tumor promoters or tumor suppressors.

In summary, through this thesis project, we first provided novel experimental evidence that BCO1/BCO2 could play a significant role in maintaining normal hepatic lipid and cholesterol homeostasis, potentially through the regulation of the FXR/miR-34a/SIRT1 pathway. Secondly, we demonstrated that BCX feeding mitigates HRCD-induced NAFLD in both WT and DKO mice through different mechanisms in the liver-mesenteric adipose tissue axis, depending on the presence or absence of BCO1/BCO2. Finally, we demonstrated that BCX feeding may alleviate HRCD-promoted HCC progression by modulating the acetylation of p53, hypoxic tumor microenvironment and glucose metabolism, independent of BCO1 and BCO2. Collectively, the findings of our studies indicate that BCX, as a dietary biological active component of sweet red pepper, plays a unique biological role in preventing the development of HRCD-induced NAFLD and HCC.

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Abbreviations

ABCG5: ATP-binding cassette transporter5 (ABCG5), **ABCG8**: ATP-binding cassette transporter8, **ACC1**: acetyl-CoA carboxylase 1, **ACC2**: Acetyl-CoA carboxylase 2, **ACOX1**: acyl-CoA oxidase 1, **AMPK**: AMP-activated protein kinase, **BCX**: β -cryptoxanthin, **BCO1**: β -carotene-15, 15'-oxygenase, **BCO2**: β -carotene-9', 10'-oxygenase, **CYP7A1**: cholesterol 7 α -hydroxylase, **CPT-1**: carnitine palmitoyltransferase-1, **DEN**: diethylnitrosamine, **DKO**: double knock out, **DNL**: de novo lipogenesis. **FA β -oxidation**: fatty acid β -oxidation, **FFAs**: free fatty acids, **FAS**: fatty acid synthase, **FXR**: farnesoid X receptor, **G6Pase**: glucose-6-phosphatase, **HCC**: hepatocellular carcinoma, **HIF-1 α** : hypoxia-inducible factor-1 α , **HO-1**: heme-oxygenase-1, **HRCD**: high-refined carbohydrate diet, **HPLC**: high-performance liquid chromatography, **HDL**: high-density lipoprotein, **HMGCR**: 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase, **HMGCS1**: HMG-CoA synthase 1, **IL-4**: Interleukin-4, **IL-6**: interleukin-6, **IL-18**: interleukin-18, **LDL**: Low-density lipoprotein, **LDHA**: lactate dehydrogenase, **MAT**: mesenteric adipose tissue, **MMP2**: matrix metalloproteinase 2, **MMP9**: matrix metalloproteinase 9, **miR**: microRNA, **NAD⁺**: nicotinamide adenosine dinucleotide, **NAFLD**: non-alcoholic fatty liver disease, **NASH**, non-alcoholic steatohepatitis, **PEPCK**: phosphoenolpyruvate carboxykinase, **PPAR α** : peroxisome proliferator-activated receptor- α , **qRT-PCR**: quantitative Real-Time PCR, **SCD-1**: stearoyl-CoA desaturase-1, **SHP**: small heterodimer partner, **SIRT1**: sirtuin1, **SNPs**: single nucleotide polymorphisms, **TG**: triglyceride, **TC**: total cholesterol, **WT**: wild-type, and **SREBF2**: sterol regulatory element binding transcription factor 2.

Chapter 1. Introduction

Background and significance

Non-alcoholic fatty liver disease (NAFLD) is the most commonly occurring chronic liver disease worldwide, and its incidence has constantly been escalating, parallel to that of obesity [1]. NAFLD is characterized by excess hepatic lipid accumulation, which in turn, can lead to inflammation (nonalcoholic steatohepatitis; NASH), and the advanced stage of the disease, cirrhosis and hepatocellular carcinoma (HCC) [2]. In the United States, HCC incidence has escalated by 80% during the past few decades, with HCC being the third most common cause of mortality. [3-5]. Considering the high prevalence and mortality of NAFLD/NASH and HCC, it is crucial to find dietary means to prevent the development of NAFLD/NASH and HCC.

Accumulating evidence from various studies has shown that high-refined carbohydrate diets (HRCD) including high-fructose and high-sugar diets are a potential risk factor for the development of NAFLD/NASH and HCC [6-8]. According to the “two-hit” hypothesis, fructose induces hepatic lipid accumulation, which makes the liver susceptible to the secondary hits such as inflammation, leading to NASH and HCC [8]. Fructose also directly induces pro-inflammatory responses, thereby activating the secondary events [8]. Previously, our laboratory demonstrated that, as compared with high-fat diet (HFD), an isocaloric-pair-fed HRCD, wherein 66.5 % of energy is provided as glucose- and fructose-producing carbohydrates including sucrose and maltodextrin, induced more severe hepatic steatosis and exacerbated the inflammatory response. Moreover, the extent of liver tumorigenesis observed in the HRCD group was comparable to that in the HFD diet group [9, 10]. In addition to environmental factors, emerging studies have suggested that genetic components are another significant contributor to the development of NAFLD/NASH and HCC [11, 12]. Therefore, it is critical to consider both genetic factors and environmental factors for understanding the pathophysiology of NAFLD/NASH and HCC.

The enzymes β -carotene-15,15'-oxygenase 1 (BCO1) and β -carotene-9',10'-oxygenase 2 (BCO2) are necessary for carotenoid cleavage to generate vitamin A and apo-carotenoids. Recent studies have suggested that these enzymes possess biological function other than just

carotenoid cleavage [13, 14]. Both BCO1 knock out (KO) and BCO2 KO studies have shown that BCO1 or BCO2 seems to regulate lipid and cholesterol metabolism in the liver [15, 16]. Moreover, BCO2 has proven to be involved in mitochondrial function and in oxidative stress regulation [14, 16]. Although single nucleotide polymorphisms (SNPs) in BCO1/BCO2 have been shown to associate with carotenoid status and some disease in humans [17-22], further research is needed to examine the effect of both BCO1/BCO2 on the development of NAFLD.

β -Cryptoxanthin (BCX) is a pro-vitamin A carotenoid primarily found in sweet red peppers, tangerines, butternut squash, and papayas [23]. BCX is preferentially cleaved by BCO1 and BCO2 to produce bioactive metabolite products such as vitamin A [23]. BCX has been shown to have many beneficial effects including anti-inflammatory, antidiabetic, anti-obesity, anti-atherosclerosis, and neuropathic pain-relieving effects [24-26]. Dietary intervention studies in rodents have shown that BCX supplementation effectively suppressed high-fat diet- or high-cholesterol diet-induced-NAFLD and NASH, by inhibiting the inflammatory response [27-29]. Nevertheless, it remains unknown whether BCX itself or its metabolites produced by cleavage enzymes are responsible for this protective effect. These studies posed the question as to whether BCX can prevent HRCD-induced-NAFLD/NASH and HCC by regulating inflammatory responses, and if the protective effect is due to the BCX itself or its metabolites generated by cleavage enzymes.

Hypothesis

In this proposed research, **we hypothesize that 1) carotenoid cleavage enzymes have unique roles in maintaining the homeostasis of lipid and cholesterol metabolism, and 2) dietary BCX itself may have protective effects against HRCD-induced NAFLD and HCC potentially independent of carotenoid cleavage enzymes.** We plan to test these hypotheses and accomplish the overall objectives by pursuing the following specific aims:

Specific aims

2.1 Specific Aim 1: To investigate the role of carotenoid enzymes (BCO1 and BCO2) other than their carotenoid cleavage, on the development of NAFLD and its underlying mechanism(s) in mice: The working hypothesis for this aim is that the BCO1 and BCO2 enzymes are necessary for maintaining the homeostasis of lipid and cholesterol metabolism, and controlling oxidative stress. We will test this hypothesis with liver tissues of wild-type and BCO1/BCO2 double knock-out (DKO) mice fed a standard chow diet. The primary outcomes will be the measure the incidence of hepatic steatosis, hepatic triglyceride and total cholesterol levels, the expressions of genes and proteins involved in lipogenesis-related enzymes, cholesterol synthesis, and oxidative stress.

2.2 Specific Aim 2: To elucidate whether the protective effects of BCX against HRCD-induced NAFLD is independent of BCO1/BCO2, and explore the underlying mechanisms in mice: The working hypothesis for this aim is that the intact BCX molecule, without being cleaved by BCO1 and BCO2, is effective in preventing HRCD-induced NAFLD. We will test this hypothesis with liver tissues of BCO1/BCO2 DKO mice, which were fed a high-refined carbohydrate diet (HRCD, 66.5 % calories from carbohydrate composed of maltodextrin and sugar), with or without BCX feeding. The primary outcomes for this aim will be the hepatic steatosis incidence, hepatic triglyceride and total cholesterol levels, the expressions of genes and proteins involved in lipogenesis, cholesterol synthesis, and oxidative stress.

2.3 Specific Aim 3: To examine whether BCX has chemo-preventative effects against diethylnitrosamine (DEN)-initiated, HRCD-promoted hepatic tumorigenesis independent of carotenoid enzymes, and explore the underlying mechanisms in hepatic tumors of mice: The working hypothesis for this aim is that the intact BCX molecule, without being cleaved by BCO1 and BCO2, can suppress liver-specific carcinogen DEN-initiated, HRCD-promoted HCC. We will investigate this hypothesis with liver tissues collected from the well-established DEN-initiated BCO1/BCO2 DKO mice fed a HRCD, with or without BCX feeding. The primary outcomes

will be the incidence of liver tumors, tumor volume, surface tumor number, and the gene and protein expression of multiple molecular pathways.

Chapter 2. Literature Review

1. Chemistry and BCX metabolism and the role of BCO1 and BCO2 beyond carotenoid cleavage activity

1.1. Chemistry and BCX metabolism

BCX is a pro-vitamin A carotenoid and one of the six most abundant carotenoids in human plasma and tissues [23]. The primary source of BCX is fruit juices; citrus fruits such as tangerines, oranges, and mandarins; papaya; and sweet red peppers [30, 31]. Two carotenoid cleavage enzymes, β -Carotene-15,15'-oxygenase (BCO1) and β -carotene-9',10'-oxygenase (BCO2), have been shown to metabolize BCX in animals and humans. BCO1 cleaves BCX at the 15,15' double bond, which is critical for vitamin A production [32], while BCO2 cleaves BCX at the 9',10' double bond to generate apo-10'-carotenoids [16]. Both BCO1 and BCO2 are highly expressed in the liver and other peripheral tissues. Clinical evidence has demonstrated that higher levels of BCX in serum are associated with lowered risk of several cancers including lung cancer [33], squamous cell carcinoma of the esophagus [34], and bladder cancer [35]. A recent clinical intervention study has shown that consumption of BCX-enriched juice with a carotenoid mixture capsule for 2.5 years led to a lowered risk of liver cancer among hepatitis patients with cirrhosis [36]. These studies raise an important question; is the protective effect of BCX against diseases, especially cancer, due to intact BCX or its metabolites such as vitamin A or apocarotenoids.

1.2. Genetic variants of BCO1/BCO2 in animals and humans

Single nucleotide polymorphisms (SNPs) of the *BCO1/BCO2* genes related to a variety of conditions have been reported in humans. Previous studies have reported that

BCO1 gene SNPs are common in humans and related to impaired catalytic activity in the bioconversion of β -carotene to vitamin A [19], although no significant association has been observed between *BCO1* SNPs and the risk of various cancers such as breast [37], prostate [37], and lung cancer [38]. A recent study has shown that *BCO1* SNPs are associated with plasma lycopene in response to the consumption of tomato juice in prostate cancer patients [39]. The Age-Related Eye Disease Study demonstrated that the *BCO2* rs2250417 SNP is strongly related to age-related eye disease [22]. *BCO2* SNPs have also been associated with alterations in the status of human and animal carotenoid levels, pro-inflammatory cytokine IL-18 expression [18], and susceptibility to ischemic stroke [40]. Higher serum levels of carotenoids have been reported to be associated with decreased risk of several cancers [41, 42]. A recent study has demonstrated that high vitamin A levels in serum were related to a lowered risk of lung and liver cancers, but increased risk of prostate cancer [43]. These studies suggest that the conflicting clinical trial results regarding the chemo-preventive effects of carotenoids might be due to the existence of *BCO1/BCO2* polymorphisms and the potential function of *BCO1/BCO2* in cancers in humans.

1.3. The role of *BCO1* and *BCO2* other than carotenoid cleavage activity

BCO1 and *BCO2* knockout (KO) mice studies indicate that these enzymes play diverse roles in many physiological processes [14, 44]. *BCO1* is involved in regulating lipid accumulation [15], insulin [45], steroid metabolism [46], and heart function [47], while *BCO2* is involved in modulating mitochondria [14, 48] and anemia [44]. Moreover, emerging cell culture studies have indicated that the *BCO1* and *BCO2* enzymes might

serve as tumor suppressors [49-51]. The authors observed that BCO1 inhibition in colon cancer cells increased certain characteristics of colon cancer including cell migration and invasion [49]. In addition, BCO1 overexpression in human neuroblastoma cells led to the suppression of cancer stemness and metastasis in human neuroblastoma cells [51]. BCO2 overexpression in prostate cancer cells also inhibited colony formation and proliferation [50]. Moreover, no significant associations between *BCO1* SNPs and various cancers risks such as breast [37], prostate [37], and lung cancer have been observed in clinical data [38]. As these studies indicate that BCO1 and BCO2 might not impact cancer development, it is possible that the beneficial effects of BCX consumption on several cancers in humans might not be affected by the presence or absence of *BCO1/BCO2* polymorphisms in humans. This hypothesis makes it more important to understand the molecular mechanisms underlying BCX prevention of the development/progression of various cancers.

2. The effects of BCX in cancer prevention

2.1. BCX in cancer prevention in animal models

Animal model studies have shown that BCX exerts protective effects against the development of several cancers including lung cancer [52-54], colon cancer [55], gastric cancer [56], and urinary bladder carcinogenesis [57], by targeting multiple molecular mechanisms. Below we summarize the major targets of BCX that can lead to suppression of the development or progression of various cancers.

2.2. Molecular mechanisms underlying BCX suppression of cancer development/progression

2.2.1. *The NF- κ B signaling pathway*

Nuclear factor- κ B (NF- κ B) is a ubiquitous nuclear transcription factor that plays a pivotal role in various pathological processes such as inflammation, cell growth and survival, immune response, and development [58, 59]. NF- κ B has been shown to activate the expression of genes involved in cell proliferation and cell survival [60]. It has been shown that tumor samples from lung cancer patients had significantly higher levels of NF- κ B in both non-small lung cancer and small cell lung cancer [61], which was correlated with disease advancement and poor prognosis of lung cancer patients [62]. Emerging pre-clinical evidence has demonstrated the tumor promoting role of NF- κ B activation in lung inflammation, and tumorigenesis [63, 64]. Several factors can activate the NF- κ B signaling pathway including tobacco-specific N-nitrosamine, cigarette smoke condensate, tumor necrosis factor (TNF)- α , and oxidative stress [65, 66]. The efficacy of current therapies for lung cancer, including systemic chemotherapy or radiation, has reached a plateau as they induce NF- κ B activation, which causes resistance to these therapies [67]. Thus, it is important to identify dietary components targeting the NF- κ B signaling pathway. Previously we showed that when we exposed male ferrets with or without BCX (10 and 20 mg/ kg diet) to smoke for 3 months, smoke alone exposure induced significantly higher levels of inflammation, oxidative stress DNA damage, and squamous metaplasia in the lung tissues, which were significantly improved by BCX supplementation in a dose-dependent manner [53]. Additionally, using immunohistochemistry analysis, we found that the protein expression levels of inflammation markers including TNF- α , NF- κ B, and

[illegible]

Figure 1. Molecular mechanisms underlying BCX prevention of cancer development/progression. (a) Smoke exposure induces oxidative DNA damage and squamous metaplasia and increases NF- κ B and AP-1 in the nucleus of ferret lung tissues. BCX supplementation improves oxidative damage, lung inflammation, and squamous metaplasia by reducing nuclear NF- κ B and AP-1 in the lung tissues; (b) BCX treatment also suppresses lung cancer development by restoring RAR β and SIRT1, which were suppressed by NNK and nicotine treatment in mice; (c) NNK treatment activates the NACHRs/PI3K/Akt pathway, which is reversed by BCX treatment in a lung cancer murine model; (d) BCX treatment regulates p73 RNA splicing, producing less oncogenic truncated Δ NP73, thereby suppressing proliferation and contributing to the inhibition of colon cancer in mice; (e) BCX increases acetylated-p53, an active form of p53, thereby inhibiting HIF-1 α , LDHA, and increasing G6Pase and PEPCK in hepatic tumors and leading to the suppression of HCC in WT and BCO1/BCO2 DKO mice; (f) BCX upregulates p53 and induces G0/G1 cell cycle arrest and apoptosis in gastric and bladder cancer in mice.

2.2.2. Retinoic acid signaling pathway

It is well established that retinoic acid (RA) is a critical bioactive metabolite of vitamin A. Importantly, RA and its derivatives has been regarded as potential chemopreventive agents owing to their ability to inhibit proliferation and induce apoptosis, differentiation, and anti-oxidant effects in cancers [68]. RA activity is mostly mediated via RA receptors (RARs) and RA X receptors (RXRs), which differ in their amino- and carboxyl-terminal domain sequence and retinoid-binding specificity [69, 70]. Downregulation of RA signaling

has been observed in several cancer cell types including head and neck [71, 72], breast [73, 74], esophagus [75], prostate [76], lung [77-79], and cirrhosis and liver cancer [80]. It has been reported that cigarette smoke reduces RAR- β 2 expression with morphological changes in the lung tissues of animals [81] and that the combination of cigarette smoke and nicotine-derived nitrosamine ketone (NNK) treatment leads to aberrant methylation of RARs in murine lung tumors [82]. Activation of the RAR signaling pathway is considered a potential therapeutic strategy for cancer prevention [83]. As the bioavailability of various natural RAR ligands (such as all-trans RA, 9-cis-RA, and 13-cis-RA) are affected by various factors, such as retinoid binding proteins involved in transport, uptake, storage, and sequestration of retinoid metabolic enzymes [84], it is important to identify natural RAR ligands other than RA to treat various chronic diseases [85]. BCX has been reported to be a novel natural RAR ligand [52, 86]. Previously, we demonstrated that BCX treatment suppressed the growth of non-small-cell lung cancer cells and immortalized human bronchial epithelial cells by up-regulating RAR β expression in a dose dependent manner [87]. BCX treatment also activated RAR specific response elements (RARE)-driven transcription activity, suggesting that the activation of the retinoid signaling pathway by BCX could be due to BCX or its metabolites [87]. Matsumoto *et al.* demonstrated that BCX itself might be a natural RAR ligand; they observed that BCX had high binding activity against RAR in an *in vitro* CoA-BAP system [85]. Previously, we have shown that BCX supplementation suppressed the development of NNK- and nicotine treatment-induced lung cancer and emphysema in mice [52]. We found that NNK and nicotine treatment impaired RAR β levels, which were restored by BCX supplementation in NNK- and nicotine treatment-induced lung cancer and emphysema in male A/J mice

[52]. Similarly, Quesada-Gómez *et al.* demonstrated that BCX inhibited angiogenesis; BCX treatment reduced vessel formation by potentially up-regulating RAR levels in mice [88]. Taken together, these results indicate that activation of RARs by BCX itself or its metabolites can contribute to suppression of cancer development, especially lung cancer (Fig. 1b).

2.2.3. SIRT1 signaling pathway

SIRT1, a nicotinic adenosine dinucleotide (NAD⁺)-dependent protein-deacetylase, plays important biological roles in several processes including circadian rhythms, lipid/glucose metabolism, inflammation, and immune function [89-91]. It has been reported that smoke exposure decreases SIRT1 levels in the nucleus [92]; this was associated with increased acetylation of nuclear RelA/p65 and IL-18 secretion [92]. Similarly, when male A/J mice were injected with NNK and nicotine treatment with or without BCX supplementation (10 mg/kg diet, 20 mg/kg diet), NNK- and nicotine treatment-induced lung cancer and emphysema were ameliorated with BCX supplementation [52]. Moreover, NNK and nicotine treatment significantly reduced the protein levels of SIRT1 in lung tissues, which recovered to normal levels following BCX supplementation in AJ mice [52]. It has been reported that SIRT1 directly controls lung inflammation [93]; SIRT1^{-/-} mice showed higher levels of inflammation after ambient particulate matter exposure, which is intimately related to increased NF-κB acetylation [93]. In agreement with this finding, we found that the reduction in SIRT1 protein levels by NNK and nicotine treatment was accompanied by elevated IL-6, which was decreased by BCX supplementation [52]. Collectively, these results suggest that BCX is a potential

dietary factor that plays an important role in restoring SIRT1 protein levels, thereby contributing to the amelioration of inflammation and the development of lung cancer and emphysema (Fig. 1b). However, SIRT1 has been reported to have dual effects on carcinogenesis depending on cancer cell lines [94]; SIRT1 has been suggested as a potential tumor suppressor in human skin tumors [95], whereas it has been suggested as a potential tumor promoter in the ovary, breast, stomach, and pancreas cancer [96-99]. The role of SIRT1 in other cancers, such as liver cancer, remains controversial [100-103]; the effects of BCX on SIRT1 protein levels in a DEN-initiated, HRCD-promoted hepatocellular carcinoma (HCC) model remains unknown. As the role of SIRT1 in HCC is controversial, future studies need to investigate whether SIRT1 acts as a tumor suppressor or tumor promoter in HCC and furthermore, how BCX differently regulates SIRT1 levels in tumors or non-tumor tissues in mice.

2.2.4. Nicotinic Acetylcholine Receptor (nAChR) $\alpha 7$ signaling pathway

Nicotine is the major component in cigarette smoke that can interact with nAChR [104-106]. Ionotropic nicotinic acetylcholine receptors (AChR) are expressed in both neuronal and non-neuronal cells. $\alpha 7$ -nAChR is a major subtype of AChR which is associated with chronic obstructive pulmonary disease (COPD) and lung cancer development [104, 106, 107]; it can react with NNK and nicotine and is a proposed molecular target [105, 108-111]. $\alpha 7$ -nAChR^{-/-} KO mice exhibited airway epithelium remodeling, namely basal cell hyperplasia and squamous metaplasia [112] and induced collagen type 1 expression following nicotine treatment, which was not observed in WT mice [113]. Antagonizing $\alpha 7$ -nAChR has been reported to inhibit lung tumor growth and tumor cell migration [109, 114].

BCX treatment inhibited cell migration and invasion of $\alpha 7$ -nAChR positive cancer cells by inhibiting the $\alpha 7$ -nAChR/PI3K/AKT signaling pathway, but not in $\alpha 7$ -nAChR negative cancer cells *in vitro* [54]. Moreover, BCX supplementation effectively reduced nicotine-promoted emphysema and NNK-induced adenocarcinoma multiplicity without significant changes in vitamin A (retinol and retinyl palmitate) levels in both mouse lungs and serums [54, 115]. We observed that BCX supplementation in mice 2 weeks prior to NNK injection effectively reduced lung tumor multiplicity by 52 – 63%, without significant changes in liver tumor incidence, suggesting that BCX was effective in suppressing tumor promotion, rather than inhibiting the initiation of lung tumorigenesis [54]. In addition, we have previously shown that the mechanisms underlying the protective effects of BCX on NNK-induced lung adenocarcinoma are associated with suppression of NNK-induced $\alpha 7$ -nAChR and phosphorylation of its down-stream target, AKT, in mouse lungs [54]. In agreement with our results, previous studies have shown that $\alpha 7$ -nAChR, in particular, was up-regulated in smokers and in smoking patients with lung cancer [116, 117]. Collectively, BCX suppress cancer cell motility and lung tumorigenesis via down-regulation of $\alpha 7$ -nAChR/PI3K/AKT signaling (Fig. 1c).

2.2.5. Tumor suppressor proteins p53 and p73

p53 is a tumor suppressor gene that plays important roles in senescence, cell-cycle arrest, and apoptosis under various cellular stress conditions [118]. Many cancers exhibit loss of p53, which contributes to chemotherapy resistance [119]; thus, re-activation of p53 is a potent strategy for treating cancer. p73, a member of the p53 transcription factor family, regulates similar target genes [120]. Several small molecules

that activate p73 have been reported to have anti-tumorigenic effects [121, 122]. p73 undergoes mRNA splicing to produce Δ Np73, an oncogenic truncated p73 that negatively regulates p53 and p73 and is responsible for chemo-resistance [123]. A recent study by San *et al.* showed that the lymphocytes of healthy volunteers who were administered a juice containing 0.75 mg of BCX for one month exhibited downregulation of Δ Np73 in their lymphocytes [55]. Additionally, they found that BCX treatment enhanced the anti-carcinogenic effects of oxaliplatin in colon cancer cell lines (HCT116, SW480) by decreasing the expression of Δ Np73 and inducing apoptosis [55]. Moreover, when they subcutaneously injected HCT116 cells into nude mice and treated them with BCX alone or BCX with oxaliplatin [55], they found that BCX alone or the combination treatment of BCX and oxaliplatin significantly suppressed tumor development along with significant inhibition of Δ Np73 levels in the kidneys, normal colonic mucosa, and xenograft tumors compared to the control group [55]. These results suggest that BCX affords protective effects against colon cancer growth by regulating the oncogenic p73 truncated isoform and might have broader effects on different tissues (Fig. 1c). Gao *et al.* have shown that BCX supplementation inhibited gastric carcinogenesis by inducing G0/G1 arrest and suppressing cyclinD, cyclinE, cyclin-dependent kinases (CDKs) of CDK4 and CDK6, potentially by increasing p53 and p21 protein levels [56] (Fig. 1f). Miyazawa *et al.* showed that BCX feeding decreased the multiplicity and incidence of preneoplastic and neoplastic regions of bladder cancer were reduced, in conjunction with inhibition of cyclin-D-positive cells [57] (Fig. 1f). Coral *et al.* also found that BCX treatment inhibited the number of colon cancer cells in the S-phase largely due to the anti-proliferative activity of BCX rather than apoptosis [55]. Anti-proliferative effects of BCX treatment have been reported in both

normal and tumor cells [87, 124]. Collectively, these results suggest that BCX might be a potential natural bioactive component that can be used in combination with conventional chemotherapeutic agents; it negatively regulates $\Delta Np73$, but activates p53 potentially by regulating post-translational modification in cancer (Fig. 1d and f). However, further studies are required before BCX can be used as a cancer treatment owing to the possibility that BCX can obstruct conventional drug treatment; thus, it is necessary to unravel the mechanisms underlying BCX regulation of p53 activity.

2.2.6. Regulation of energy metabolism in tumors

Cancer cells tend to have altered energy metabolism, known as the Warburg effect, which is characterized by higher glucose uptake and glycolysis rather than oxidative phosphorylation [125]. The Warburg effect has been widely examined in human cancers, including HCC [126], and is closely associated with the aggressiveness and high proliferation of the cancer [127, 128]. Lactate dehydrogenase (LDHA) is an important enzyme regulating glycolysis and HCC proliferation [129], and targeting glucose metabolism is a promising strategy for treating HCC. Based on this hypothesis, Thus, it would be interesting to investigate whether BCX supplementation modulates glucose metabolism in the tumors, which may contribute to the inhibition of cancer progression.

2.2.7. Lipid droplets, cancer stem cells, and niche in cancer

It is well-known that obesity is a major risk factor for several cancers [130]; however, the underlying mechanisms remain unclear. Accumulating evidence in recent years has drawn attention to the role of lipid droplets in cancer development [131-133]; lipid-rich

cancer cells exhibit more aggressive behavior and chemotherapy resistance in animal and human studies [131-133]. Cancer cell reprogrammed energy metabolism not only induces glycolysis, but also increases *de novo* lipogenesis [134, 135]. Lipid droplets are intimately related to inflammation, which can lead to the development and progression of neoplastic regions [136]. Cancer stem cells (CSCs) are a subset of cells that have stem-like characteristics in tumors that are responsible for self-renewal, tumor relapse, and drug resistance [137]. CSCs also demonstrate energy metabolism alteration including higher glycolytic rates, lower mitochondrial respiration, and higher lipid synthesis [138]. It has been shown that saturated fatty acids can activate NF- κ B [139] and that activation of the NF- κ B signaling pathway can increase inflammatory and cancer stem cell markers in CSCs [140]. CSC niche, including nutrient supply and hypoxic tumor microenvironment, play important roles in regulating CSCs. A hypoxic tumor microenvironment triggers cancer cells to uptake fatty acids from the outside [141]. Moreover, cancer cells can uptake lipids secreted by extra-vesicles secreted from nearby hypoxic cells [142]. In addition, it has been directly demonstrated that fatty acids translocated from adipose tissue are incorporated by cancer cells [143].

β -Carotene, another pro-vitamin A carotenoid, has been shown to have the ability to inhibit neuroblastoma tumorigenesis by modulating cancer stem cell markers including Oct3/4 and delta-like 1 homologue (DLK1) and hypoxic tumor microenvironment marker HIF-1 α in neuroblastoma tumors [144]. Moreover, β -carotene effectively regulated the levels of DLK1 and epithelial mesenchymal transition (EMT), antioxidant enzymes in the normal tissue surrounding tumor tissues, suggesting that β -carotene affects tumor microenvironment unfavorably for tumor growth [145]. However, the relationship between

BCX and CSCs is unknown; thus, it is necessary to investigate whether BCX can regulate cancer stemness in the tumors by modulating the CSCs niche such as lipid accumulation and inflammation.

Chapter 3. Project 1.

: To investigate the role of carotenoid enzymes (BCO1 and BCO2) other than their carotenoid cleavage, on the development of NAFLD and its underlying mechanism(s) in mice

This study has been published

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Ablation of carotenoid cleavage enzymes (BCO1 and BCO2) induced hepatic steatosis by altering the farnesoid X receptor/miR-34a/sirtuin 1 pathway.

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ABSTRACT

β -Carotene-15, 15'-oxygenase (BCO1) and β -carotene-9', 10'-oxygenase (BCO2) are essential enzymes in carotenoid metabolism. While BCO1/BCO2 polymorphisms have been associated with alterations to human and animal carotenoid levels, experimental studies have suggested that BCO1 and BCO2 may have specific physiological functions beyond the cleavage of carotenoids. In the present study, we investigated the effect of ablation of both BCO1/BCO2 in the development of non-alcoholic fatty liver disease (NAFLD) and its underlying molecular mechanism(s). BCO1/BCO2 double knock out (DKO) mice developed hepatic steatosis (8/8) and had significantly higher levels of hepatic and plasma triglyceride and total cholesterol compared to WT (0/8). Hepatic changes in the BCO1/BCO2 DKO mice were associated with significant: 1) increases in lipogenesis markers, and decreases in fatty acid β -oxidation markers; 2) upregulation of the cholesterol metabolism markers; 3) alterations to the microRNAs related to TG accumulation and cholesterol metabolism; 4) increases in hepatic oxidative stress marker (HO-1) but decreases in anti-oxidant enzymes; and 5) decreases in farnesoid X receptor (FXR), small heterodimer partner (SHP), and sirtuin 1 (SIRT1). The present study provided novel experimental evidence that BCO1/BCO2 could play a significant role in maintaining normal hepatic lipid and cholesterol homeostasis, potentially through the regulation of the FXR/miR-34a/SIRT1 pathway.

Key words: fatty liver disease; carotenoid cleavage enzymes; Farnesoid X receptor;

Sirtuin 1

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1. INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD), the most common form of chronic liver disease [146], has dramatically increased in the last 40 years and has been associated with the increased prevalence in obesity [147] of the general population. The spectrum of NAFLD is broad and varies from simple hepatic triglyceride accumulation to non-alcoholic steatohepatitis (NASH) which can progress to terminal hepatic diseases such as cirrhosis or liver cancer [148]. 'Lean NAFLD' (NAFLD in the absence of obesity) has been described in at least 5% of the US population and has been identified in most western countries [1]. Multiple etiological factors include: high fat and fructose intake and genetic predisposition/risk factors have been shown to contribute to lean NAFLD [1]. Numerous single nucleotide polymorphism (SNP) variants associated with lipid metabolism, inflammation, oxidative stress, and insulin resistance have been suggested as potential genetic factors contributing to the development of NAFLD [11]. Thus, it is critical to identify the molecular mechanisms and potential genetic variants that may result in a susceptibility to NAFLD to understand the potential for the prevention and treatment of the development of NAFLD.

In mammalian tissues, β -carotene-15,15'-oxygenase (BCO1) cleaves carotenoids such as β -carotene and β -cryptoxanthin at the 15,15' double bond which is essential for vitamin A production and homeostasis [149]. β -Carotene-9',10'-oxygenase (BCO2) cleaves carotenoids at the 9,10 double bond to generate the biologically active apo-10'-carotenoids [150]. Previous studies have suggested that SNPs in the human *BCO1* gene are common and are associated with reduced catalytic activity in the conversion of β -carotene to vitamin A [19, 151]. The rs6564851 SNP of BCO1 is particularly critical for those at risk of vitamin A deficiency [152]; further, this has been associated with high levels of plasma HDL in healthy subjects [153], although there was no association observed between SNP in BCO1 and risk of breast cancer [37], and type 2 diabetes [21]. The Carotenoids in Age-Related Eye Disease Study (CAREDS) has

reported that the rs2250417 SNP in BCO2 was strongly related to age-related eye disease [22]. The rs2115763 SNP of BCO2 was associated with interleukin-18 (IL-18) concentrations [18].

BCO1 and BCO2 knockout (KO) mice present phenotypes, in addition to accumulation of carotenoids in tissues, suggesting that these enzymes likely have broader roles resulting in a myriad of physiological changes [14, 44]. Both BCO1 and BCO2, with different subcellular localization, are highly expressed in the liver and other peripheral tissues [154, 155]. Studies using BCO1 KO mice indicate that BCO1 has a role in the development of hepatic steatosis [15], and in obesity [15], lipid metabolism [15], heart metabolism and function [47], serum insulin, leptin, cholesterol [45], steroid metabolism [46], testosterone synthesis and prostatic cellular proliferation [13]. BCO2 ablation has been shown to result in mitochondrial dysfunction, oxidative stress [14, 48], anemia [44], metabolic alteration, liver steatosis [16], and prostate cancer progression [50]. It has been reported that ablating BCO1 has resulted in over-expression of BCO2 [156, 157], and carotenoid accumulation influenced whole-body respiration and energy expenditure in the absence of both BCO1 and BCO2 [45, 158]. In the present study, we examined if the hepatic and extra-hepatic phenotype resulting from the lack of both BCO1 and BCO2 contributes to NAFLD development.

The nuclear hormone farnesoid X receptor (FXR), which is mainly expressed in the liver, has been shown to regulate lipogenesis, cholesterol homeostasis, and oxidative stress by controlling downstream gene expressions [159-161]. Constitutively, active FXR leads to improvement of hyperlipidemia and hyperglycemia [159]. Sirtuin1 (SIRT1), a NAD⁺-dependent histone/protein deacetylase, has been demonstrated to be involved in the regulation of the development of liver steatosis, oxidative stress, and insulin resistance [162, 163]. MicroRNAs (miRNAs), known as endogenous small noncoding RNA, are involved in the post-transcriptional regulation of gene expression [164, 165] and are aberrantly expressed in many diseases including NAFLD [166]. MicroRNA-34a (miR-34a) is the most abundant hepatic miRNA found in obese mice and NAFLD patients [164, 165] and has emerged as an important regulator of SIRT1. Recently,

a new regulatory network, the FXR/miR-34a/SIRT1 pathway, has been proposed [167]. The activation of this pathway has been shown to be impaired in obese mice and is associated with a marked increase in hepatic lipid accumulation and liver steatosis [167], suggesting FXR/miR-34a/SIRT1 pathway as a potential novel target for the prevention and treatment of NAFLD; whether BCO1/BCO2 affects this pathway thereby contributing to NAFLD development is unclear.

In this present study, we utilized WT and BCO1/BCO2 double KO (DKO) mice fed a standard-chow diet to examine the genetic impact of BCO1 and BCO2 enzymes on the development of NAFLD, oxidative stress, lipid metabolism and the FXR/miR-34a/SIRT1 pathway.

2. MATERIAL AND METHODS

2.1 Animals and diets

BCO1^{-/-}/BCO2^{-/-} double knockout mice with C57/BL6;129Sv mixed genetic background were provided by Dr. J. von Lintig at Case Western Reserve University [158]. Mice were tested with the Mouse 384 SNP panel (Jackson Laboratories, Bar Harbor, ME) to determine C57BL genetic background and were backcrossed onto a C57BL6J background to generate BCO1^{+/-}/BCO2^{+/-} heterozygous mice (F1 generation). The F1 were used for breeding to generate both BCO1^{-/-}/BCO2^{-/-} DKO (BCO1/BCO2 DKO) and their corresponding wild type (WT) littermates for the study. All experimental protocols and procedures were approved by the Tufts University Institutional Animal Care and Use. Mice were fed chow diet (Teklad 2916.15, Envigo, Madison, WI) and water ad libitum, maintained under conditions at 22 ± 2°C, 40 ± 5% humidity with 12/12 light/dark cycle. During the experimental period, all animals were weighed twice a week and food intake monitored. At the end of the 24-week experimental trial, all mice were terminally exsanguinated (non-fasted) under deep isoflurane anesthesia (Henry Schein, Dublin OH). Liver tissues were harvested post-mortem and weighed. The left lobe of mouse liver was fixed in 10% buffered formalin solution (Thermo Fisher Scientific, USA). The remaining sections of liver were divided into smaller portions, snap-frozen in liquid nitrogen and stored at - 80°C for subsequent analysis.

2.2 Liver histopathology evaluation

Five-micrometer sections of formalin-fixed and paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E, Sigma Aldrich, St Louis, MO, USA) for histopathological analysis, as previously described [168, 169]. Sections were examined under light microscopy by two independent investigators who were blinded to the treatment groups. A ZEISS microscope with a PixeLINK USB 2.0 (PL-B623CU) digital Camera and PixeLINK μ Scope Microscopy Software was used for image capture and quantification for all histopathological analyses. Hepatic steatosis was graded according to steatosis magnitude (both macro- and micro-vesicular fat accumulation). Briefly, the degree of steatosis was graded based on the percentage of the liver section that was occupied by fat vacuoles at 100 $\times$ magnification in 20 fields (grade 0 $\leq$ 5%; grade 1 = 5 to 25%; grade 2 = 26 to 50%; grade 3 = 51 to 75%; grade 4 = 76% to 100%). Hepatic steatosis incidence was determined by the percentage of mice with a hepatic steatosis score larger than 0.

2.3 Reverse transcription-PCR and quantitative RT-PCR

Total RNA was prepared from 50-100 mg samples of frozen liver tissue using a TriPure Isolation Reagent kit (Roche, Indianapolis, IN, USA). Briefly, deep-frozen liver tissues were minced and homogenized with Tripure Isolation Reagent (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. cDNA was synthesized by reverse transcription using M-MLV (Invitrogen, Carlsbad, CA, USA) and was used for quantitative real-time PCR analysis. Quantitative real-time PCR was performed using specific primers and FastStart Universal SYBR Green Master (Roche, Indianapolis, IN, USA). The relative expression of all mRNAs were calculated using the comparative threshold cycle (Ct) method [170]. For the normalization, the amplification of β -actin was used for loading control. The primers used were listed in supplementary table 1.

2.4 MicroRNA quantification by real-time qRT-PCR analysis

Total RNA (100 ng) was prepared from liver tissues to generate cDNA by reverse transcription with Moloney murine leukemia virus RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The relative expressions of all microRNAs were quantified using the comparative threshold cycle (Ct) method as identified above. For the normalization, the amplification of U6 for every sample was used.

2.5 Western blot

Western blotting was performed as previously described [171]. Briefly, the protein was extracted from 50-100 mg samples of liver tissue by lysing with RIPA lysis buffer; homogenates centrifuged at 55,000 rpm for 60 min at 4 °C using an Ultracentrifuge and supernatants collected and stored at – 80 °C. Equal aliquots of sample (50 µg) were used and mixed with 2 × reducing Laemmli sample buffer. Denatured protein was applied to 10% SDS-polyacrylamide gels, transferred electrophoretically to Immobilon-P membranes (Millipore, Billerica, MA, USA) and subsequently blocked with 5% non-fat dried milk in Tris-buffered saline containing Tween-20 (TBST) buffer. Membranes were probed with primary antibodies against FXR (GeneTex, Irvine, CA, USA), HO-1 (Santa Cruz, TX, USA), SCD-1, ACC (Cell Signaling, Danvers, MA, USA), and β-actin (Sigma-Aldrich) at 4 °C overnight. After being washed three times with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA) in 5% non-fat milk. All membrane-bound primary antibodies were examined by a SuperSignal West Chemi-luminescent Substrate Kit (Pierce, Rockford, IL, USA) and the specific relative band intensities were quantified by scanning with a model GS-710 Calibrated Imaging Densitometer (Bio-Rad). For normalization, β-actin was used as loading control.

2.6 Liver and plasma triglyceride (TG) and total cholesterol (TC) analysis

Hepatic and plasma TG and TC levels were measured using a commercial total cholesterol assay kit (Cell Biolabs Inc, San Diego, CA, USA) and TG colorimetric assay kit (Cayman, Ann Arbor, Michigan, USA). All procedures followed manufacturer's protocols. TG and TC concentrations were measured at 540 nm.

2.7 Statistical analysis

All results were represented as the mean $\pm$ standard error of the mean (SEM). The two-tailed, unpaired Student's t-test was used to compare TG, TC, gene and protein expression between two groups. The Mann-Whitney test was used for the liver steatosis score distribution comparison. The analysis was conducted using GraphPad-Prism 7.0 software (GraphPad Software, Inc.) and $P < 0.05$ was considered significantly different.

3. RESULTS

3.1 Ablation of BCO1 and BCO2 increases lipid accumulation in the liver

There were no significant differences in final body weight, liver weight, and mesenteric fat weight between WT and BCO1/BCO2 DKO mice (Table 1). Histological analysis revealed that 100% (8/8) of mice in the BCO1/BCO2 DKO group developed hepatic steatosis (score 1), whereas hepatic steatosis was not observed in WT animals (0/8) after the 24 week trial ($P < 0.001$) (Table 1). Liver histopathological examination demonstrated that large and small lipid vacuoles were markedly increased in the BCO1/BCO2 DKO mice ($P < 0.001$) compared to WT mice (Fig. 1). To confirm these histological findings, TG and TC levels in both liver and plasma were examined: BCO1/BCO2 DKO mice had approximately 50% higher hepatic TG ($P < 0.05$) and 75 % higher hepatic TC ($P < 0.01$) levels compared to the WT mice (Table 1). Although there were no changes in plasma TG, the plasma TC levels were significantly 54% higher in BCO1/BCO2 DKO mice ($P < 0.01$) (Table 1). Both hepatic TG and TC levels were significantly and positively correlated with liver weight/b.w ($R^2 = 0.316$, $P = 0.012$). These results indicate that both BCO1

and BCO2 genes have a pivotal role in the regulation of lipid (TG and TC) accumulation in the liver.

3.2 Ablation of BCO1 and BCO2 increases *de novo* hepatic lipogenesis and inhibits lipid β -oxidation

We examined the gene expression of mRNA and proteins related to lipogenesis and fatty acid β -oxidation in WT and BCO1/BCO2 DKO mice. We found that the stearoyl-CoA desaturase 1 (SCD1) and acetyl-CoA carboxylase (ACC) gene expression in the liver were significantly increased by 57% and 17%, respectively, in BCO1/BCO2 DKO mice compared to the WT mice ($P < 0.05$) (Fig. 2A); we confirmed that the relative protein levels of SCD1 by western blot were approximately 9-fold higher in BCO1/BCO2 DKO mice than those in WT mice ($P < 0.05$) (Fig. 2B). Further, ACC protein levels were approximately 2-fold higher in BCO1/BCO2 DKO mice compared to the WT mice ($P < 0.01$) (Fig. 2C). Hepatic gene markers for fatty acid oxidation, such as carnitine palmitoyltransferase 1 (CPT1) and peroxisome proliferator-activated receptor alpha (PPAR α), were significantly inhibited by 90 %, and 22 % respectively ($P < 0.05$) (Fig. 2A). Hepatic mRNA levels of CD36 (involved in fatty acid uptake) were up-regulated 2.5-fold in BCO1/BCO2 DKO mice ($P < 0.001$) (Fig. 2A). These data indicate that the hepatic lipid accumulation in the BCO1/BCO2 DKO mice is associated with elevated *de novo* hepatic lipogenesis and decreased fatty acid β -oxidation.

3.3 Ablation of BCO1 and BCO2 alters cholesterol metabolism

To explain the high TC levels in BCO1/BCO2 DKO mice, we investigated markers related to cholesterol metabolism. qPCR results revealed that mRNA levels of cholesterol 7 α -hydroxylase (CYP7A1) and sterol 12 α -hydroxylase (CYP8B1), involved in bile acid synthesis from cholesterol, were significantly increased in BCO1/BCO2 DKO mice ($P < 0.05$) (Fig. 3). Furthermore, gene

expression of hepatic 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoAR), a rate-controlling enzyme of cholesterol synthesis, in BCO1/BCO2 DKO mice increased by 40 % compared to the WT mice ($P < 0.001$) (Fig. 3). These data suggest that the BCO1/BCO2 DKO induced cholesterol synthesis by elevating HMG-CoAR and cholesterol utilization by CYP7A1 and CYP8B (which requires cholesterol as the precursor molecule for bile acid synthesis).

3.4 Ablation of BCO1 and BCO2 induces oxidative stress in the liver

Because accumulated lipid droplets in the liver can induce mitochondrial oxidative stress [172], we examined the anti-oxidant enzymes in the liver of the BCO1/BCO2 DKO. There was a 3-fold up-regulation of protein expression of hepatic heme-oxygenase 1 (HO-1) ($P < 0.01$) (Fig. 4A) and a 2-fold induction of HO-1 mRNA expression ($P < 0.001$) (Fig. 4B) in BCO1/BCO2 DKO mice compared to WT mice. A small but significant reduction in mRNA levels of glutathione peroxidase 1 (GPX), superoxide dismutase 1 (SOD1), SOD2, and catalase (16%, 6%, 13%, and 18%, respectively) was observed in the BCO1/BCO2 DKO mice ($P < 0.05$) (Fig. 4B). These results demonstrated that the BCO1/BCO2 DKO in these mice resulted in an increased oxidative stress and decreased antioxidant ability.

3.5 BCO1/BCO2 DKO causes impairment of the FXR/miR-34a/Sirt1 pathway in the liver

Activation of FXR induces expression of a small heterodimer partner (SHP) that results in suppression of miR-34a, thereby increasing SIRT1 levels [167]. Protein and mRNA FXR levels were significantly reduced 20 % and 17 %, respectively in BCO1/BCO2 DKO mice ($P < 0.01$) (Fig. 5A, B). Further, gene expressions of SHP and SIRT1 were decreased by 32 %, and 20 %, respectively ($P < 0.05$) (Fig. 5B). miRNA-34a was 2-fold higher in BCO1/BCO2 DKO mice ($P < 0.01$) (Fig. 5C). These data suggest that the absence of BCO1/BCO2 induced NAFLD in the DKO mice by impairment of the FXR/miR-34a/SIRT1 signaling pathway.

3.6 Ablation of BCO1 and BCO2 leads to the aberrant levels of the miR-33/miR-122 axis

MiR-122 and miR-33 are involved in *de novo* lipid synthesis, cholesterol and bile acid homeostasis, and oxidative stress [173-175]. It has been reported that miR-122 and miR-33 are downstream-targets of FXR since they have specific binding sites for FXR [176, 177]. We quantified the levels of miR-33 and miR-122 in the liver by qPCR, and observed that the BCO1/BCO2 DKO mice had a 23 % decrease in hepatic miR-122a ($P < 0.05$) (Fig. 6). In addition, hepatic levels of miR-33 in the BCO1/BCO2 DKO mice were 30 % lower than those in the WT mice ($P < 0.05$) (Fig. 6). This decrease of miR-33 and miR-122 levels (as the downstream-targets of FXR) in BCO1/BCO2 DKO mice could result from the impairment of the FXR signaling pathway.

4. DISCUSSION

In this current study, we demonstrated that the absence of BCO1 and BCO2 in mice fed a normal chow diet altered normal lipid and cholesterol metabolism and induced oxidative stress. This observation was associated with the alteration of FXR/miR-34/SIRT1 pathway and miR-33/miR-122 axis in the liver and contributing to NAFLD development. Our findings support the notion that BCO1 and BCO2 enzymes have separate biological functions from carotenoid metabolism and suggest the possibility that genetic BCO1/BCO2 polymorphism in humans could be an independent risk factor for the susceptibility to NAFLD.

In the current study, we demonstrated that all BCO1/BCO2 DKO mice developed hepatic steatosis when fed a standard chow diet for 24 weeks. In addition to histological changes, there was an increase in hepatic TG, hepatic TC, and plasma TC levels in the mice which may be a result of the combination of both enzyme deficiencies since BCO1 and BCO2 regulate lipid metabolism differently via different regulatory networks [14-16, 45]. Hessel *et al.* have demonstrated the development of marked liver steatosis with high hepatic TG in the BCO1 KO mice, suggesting that liver steatosis was directly associated with BCO1 deficiency [15]. Ford *et al.* have reported that BCO2 KO mice showed higher levels of hepatic cholesterol and total hepatic

lipid as compared to WT mice, while no differences were observed in the BCO1 KO mice [45]. Further, histopathological data indicated that hepatic steatosis was observed in 25% of the BCO1 KO mice, but not in the livers of WT or BCO2 KO mice [45]. Elevated oxidative stress markers and dysregulated mitochondrial enzyme activity have been reported in BCO2 KO mice [14]. In the current study, all BCO1/BCO2 DKO mice exhibited both the pathological characteristics previously described for BCO1 and BCO2 KO mice with more severe histopathological features (e.g. 100% of BCO1/BCO2 DKO mice developed liver steatosis) suggesting that deletion of both BCO1 and BCO2 enzymes can inevitably lead to NAFLD.

Impairment of the FXR/miR-34/SIRT1 pathway potentially mediates the development of liver steatosis by increasing lipogenesis and reducing fatty acid β -oxidation. FXR has been shown to inhibit expression of FAS, SCD-1 and ACC, and prevent overproduction of TG, suggesting a significant effect on lipogenesis [178]. Further, FXR has the ability to enhance TG clearance by inducing PPAR α and its target gene, CTP-1 [179]. We found elevated levels of hepatic lipogenesis markers (including SCD-1 and ACC) in the BCO1/BCO2 DKO mice but levels of fatty acid β -oxidation markers (including PPAR α and CPT1) were reduced. This is consistent with the reports of others that demonstrated decreased levels of markers related to fatty acid β -oxidation including PPAR α in BCO2 KO mice [14]. CD36 is a fatty acid translocase intimately related to hepatic steatosis, hepatic cholesterol uptake and highly expressed in NAFLD patients [180] and Hessel *et al.* reported that CD36 expression was increased in BCO1 KO mice [15]. In the current study, we observed an increase in hepatic CD36 expression with decreased FXR levels in the BCO1/BCO2 DKO mice. Since FXR has been demonstrated to inhibit liver steatosis by effectively reducing CD36 expression [181], our results suggest that increased CD36 expression may result from decreased hepatic FXR levels in the DKO mice. Further, CD36 overexpression in peripheral tissues reduces plasma fatty acid (FA) and TG levels [182] and we observed that increased CD36 mRNA expression was associated with higher hepatic TG levels ($P < 0.05$) and lower plasma TG levels ($P = 0.08$) in the BCO1/BCO2 DKO mice. We have previously reported that ablation of

SIRT1 activity in mice resulted in hepatic steatosis by over-expression of CD36 in the liver [171], suggesting that increased CD36 levels may also be due to suppressed SIRT1 levels in BCO1/BCO2 DKO mice. Further, we previously demonstrated that hepatic steatosis induced by a highly refined carbohydrate diet was mediated by the dysregulation of the miR-34a/SIRT1 axis in mice [183]. We found that the down-regulation of FXR was accompanied with lower expressions of SHP and SIRT1 but higher expression of miR-34a in the BCO1/BCO2 DKO mice, suggesting that both BCO1 and BCO2 play significant roles in regulating fatty acid uptake, *de novo* fatty acid synthesis, and mitochondrial fatty acid β -oxidation via the FXR/miR-34/SIRT1 pathway. The mechanism(s) underlying the suppression of FXR by BCO1/BCO2 DKO remains unclear, and further studies are needed.

In the current study, both hepatic and plasma TC accumulation were increased in the BCO1/BCO2 DKO mice. The rapid increases in cholesterol levels as NAFLD progressed to NASH suggests that TC contributes to NAFLD pathogenesis [184]. Cholesterol homeostasis is maintained by the balance of cholesterol synthesis with cholesterol efflux (by bile acid synthesis). Our findings indicate that despite a minor elevation in cholesterol utilization by CYP7A1 and CYP8B1, dramatic increases in cholesterol synthesis by HMG-CoA reductase led to the accumulation of TC in both liver and plasma in the DKO mice. These effects are also potentially mediated by the FXR/miR-34/SIRT1 pathway: FXR suppresses CYP7A1 and CYP8B1 (by induction of SHP) to control cholesterol and bile acid levels [185]; therefore, reduced expression of FXR and SHP may account for increased levels of CYP7A1 and CYP8B1 that led to increases in both hepatic and plasma TC levels in the DKO mice. Further, we observed that HMG-CoA reductase levels were significantly higher in the livers of the DKO mice. A possible mechanism for the elevation of HMG-CoA reductase in the BCO1/BCO2 DKO mice may be the elevation of hepatic miR-34a levels (Fig. 2C). miR-34, a microRNA that is highly expressed in NASH [165], inhibits SIRT1, which can subsequently lead to inactivation of 5'-prime-AMP-activated protein kinase (AMPK), a known inhibitor of HMG-CoA reductase [186, 187]. Taken together, our results

provide compelling evidence that the cooperative actions of BCO1 and BCO2 enzymes play a role in the maintenance of cholesterol homeostasis in liver and plasma by regulating expressions of HMG-CoA reductase, CYP7A1, and CYP8B1 by the FXR/miR-33/SIRT1 pathway.

To further examine the down-stream effectors of FXR, we demonstrated that the levels of miR-33 and miR-122 were significantly reduced in the livers of BCO1/BCO2 DKO mice. Others have reported the FXR-enhanced transcription of miR-33 and miR-122 by the direct binding to the specific region of miR-33 and miR-122 [176, 177], implying that impaired FXR levels were directly linked to the diminished miR-33/miR-122 axis. Although miR-33 has been reported to repress major genes in cholesterol export and high-density lipoprotein (HDL) metabolism in short-term studies, a recent study has commented that long-term inhibition of miR-33 led to high TG and liver steatosis in obese mice fed a high fat diet [188]; similarly, Horie *et al.* reported that miR-33 KO mice exhibited fatty liver, high TG levels, and obesity [189]. In the current study, we confirmed the finding that miR-122 was suppressed in BCO1/BCO2 DKO mice. Consistent with our results, Hsu *et al.* demonstrated that miR-122 KO mice exhibited liver micro-steatosis with 2-fold higher TG levels compared to the control [190]. Others have reported that miR-122 inhibition reduced serum cholesterol and lipogenesis [173, 191]. It is important to note that transient suppression of miR-122 using antisense oligonucleotides are well tolerated, while prolonged miR-122 deletion leads to hepatic TG accumulation [190]. Inactivation of FXR may lead to prolonged suppression of miR-122 with the development of NAFLD. Altogether, FXR directly or indirectly modulates genes involved in lipid synthesis, lipid β -oxidation, cholesterol metabolism, and oxidative stress by the miR-33/miR-122 axis and induces the development of NAFLD in the BCO1/BCO2 DKO mice.

Oxidative stress exacerbates the pathogenesis of NAFLD and is considered to be the most critical mechanism contributing to hepatic injury in NAFLD [192]. HO-1, a key rate-limiting enzyme involved in the conversion of heme to iron, carbon monoxide (CO), and bilirubin [193] is known to be induced by environmental stimuli such as reactive oxygen species (ROS), cytokines,

and endotoxin [194]. In the present study, both gene and protein expressions of HO-1 were augmented in the BCO1/BCO2 DKO mice with the resultant development of hepatic steatosis. Consistent with our findings, Malaguarnera *et al.* reported induced HO-1 expression subsequent to disease progression from normal/ simple steatosis to NASH [195]. A study by Jais *et al.* reported improvement to hepatic steatosis and TG levels in HO-1 KO mice fed a high-fat diet, suggesting that hepatic HO-1 was a potential predictor of disease severity [196]. The possible mechanism responsible for increased HO-1 could be related to reduced miR-122 and impaired FXR signaling in the BCO1/BCO2 DKO mice. Since the inhibition of miR-122 up-regulates HO-1 mRNA expression in hepatocytes [175], reduced miR-122 may lead to increased HO-1 levels in these mice. Consistent with our findings, the recent data by Wu *et al.* demonstrated that BCO2 KO mice exhibited higher oxidative stress with higher ROS levels, mitochondrial dysfunction, and suppression of antioxidant enzymes including SOD2 and glutathione-disulfide reductase (GSR) [14]. These results support the hypothesis that the cooperative role of BCO1 and BCO2 enzymes may work cooperatively to affect the oxidative stress by regulating HO-1, SOD1, SOD2, GPX, and catalase by miR-122 (which is regulated by the FXR pathway) in the development of NAFLD.

5. CONCLUSIONS

The current study provided strong experimental evidence that the ablation of both BCO1/BCO2 can lead to the development of NAFLD, thus suggesting that BCO1/BCO2 may play a significant role in maintaining normal hepatic lipid and cholesterol homeostasis, potentially through the regulation of the FXR/miR-34a/SIRT1 pathway. Our results furthered the growing body of evidence demonstrating that BCO1 and BCO2 have multiple roles in addition to their carotenoid cleavage activity. Further, our results emphasized the necessity of investigation of the relationship between genetic BCO1/BCO2 polymorphisms in humans and the metabolic diseases including NAFLD.

Table 1. Primary outcomes

Experimental group	WT	BCO1/BCO2 DKO	<i>P</i> -value
Mice, n	12	11	
Body weight (g)	33.15 ± 2.63	37.27 ± 6.80	0.065
Liver weight (g)	1.47 ± 0.14	1.87 ± 0.75	0.086
Liver weight/Body weight, %	4.44 ± 0.26	4.49 ± 0.99	0.14
Mesenteric fat weight (g)	0.46 ± 0.081	0.56 ± 0.24	0.199
Mesenteric fat weight/Body weight, %	1.37 ± 0.16	1.4 ± 0.38	0.53
Liver steatosis incidence, % of mice	0 % (0/8)	100% (8/8)**	<0.001
Hepatic steatosis score, median (range)	0 (0-0)	1 (1-1)**	<0.001
Hepatic lipids (mg/g tissue)			
TG level (mg/g)	7.08 ± 1.94	10.64 ± 3.99*	<0.05
TC level (mg/g)	59.42 ± 3.61	100.7 ± 15.98*	<0.05
Plasma lipids (mg/g)			
TG level (mg/g)	113.1 ± 22.08	95.1 ± 16.01	0.082
TC level (mg/g)	91.41 ± 4.31	133.1 ± 7.26*	<0.05

Values are means ± S.E.Ms. Statistical significance was determined by the unpaired t-test. To compared liver steatosis score, Mann-Whitney test was used. Value are means ± S.E.M. * *P* < 0.05; ** *P* < 0.01.

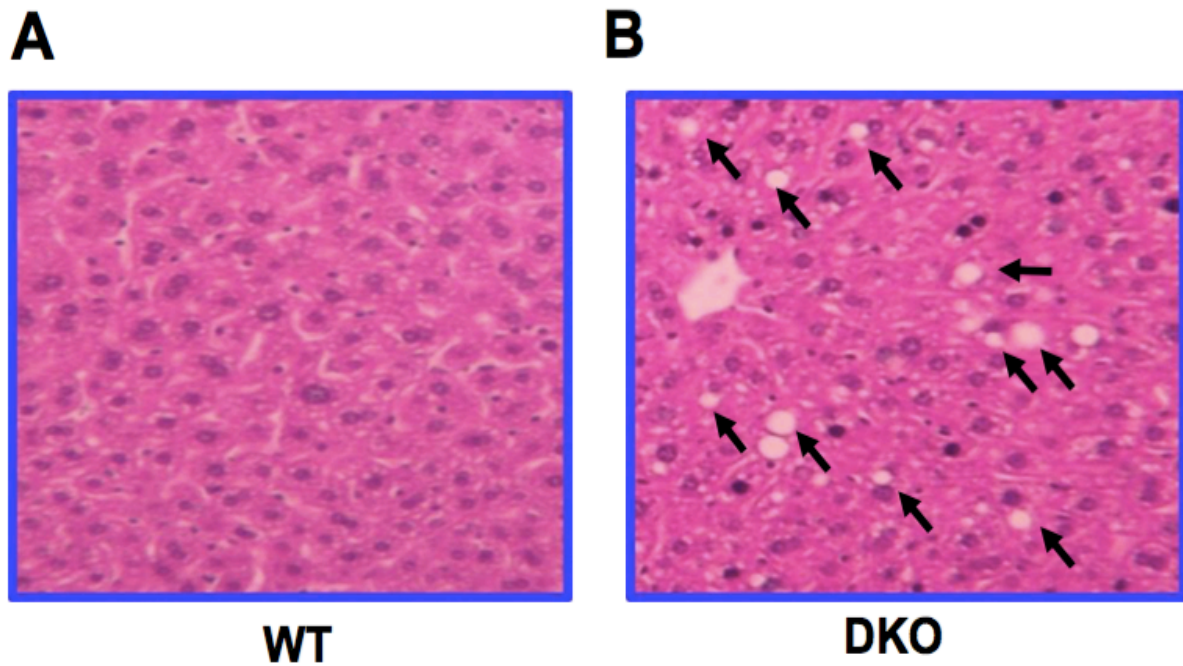


Fig. 1. Hepatic steatosis in BCO1/BCO2 DKO mice. (A) WT, (B) BCO1/BCO2 DKO.

Hematoxylin and eosin stained slides were photographed at a magnification of 100x.

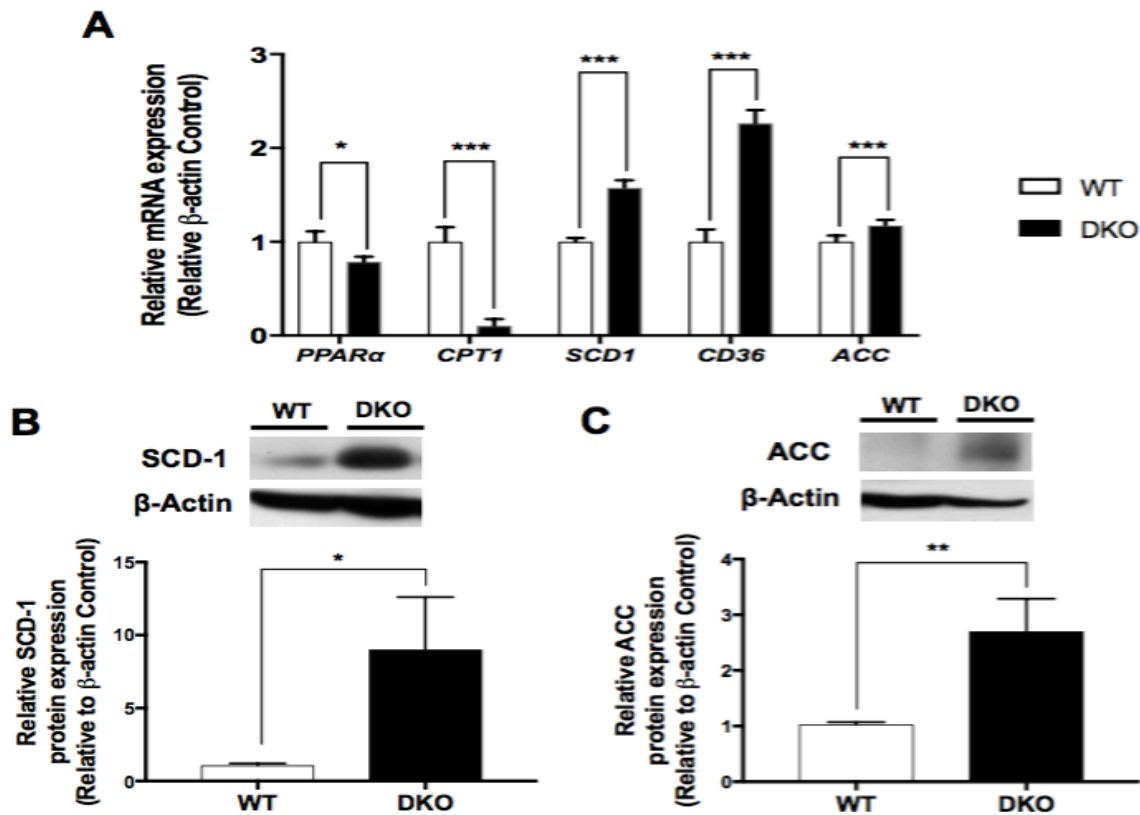


Fig. 2. The effects of BCO1/BCO2 DKO on lipogenesis and fatty acid β -oxidation biomarkers. (A) Relative mRNA levels of fatty acid synthesis markers (*SCD1*, *ACC*, *CD36*) and fatty acid β -oxidation markers (*PPAR α* , *CPT1*) were detected by qPCR in the livers of WT and BCO1/BCO2 DKO mice. β -actin was used as a loading control. (B) Relative SCD1 protein levels, (C) Relative ACC protein levels were analyzed by western blotting of liver lysates of WT and BCO1/BCO2 DKO mice. Representative results are shown in upper panels and band intensities were quantified by densitometry. β -actin was used as a loading control. Statistical significance was determined by the unpaired t-test. Values are mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

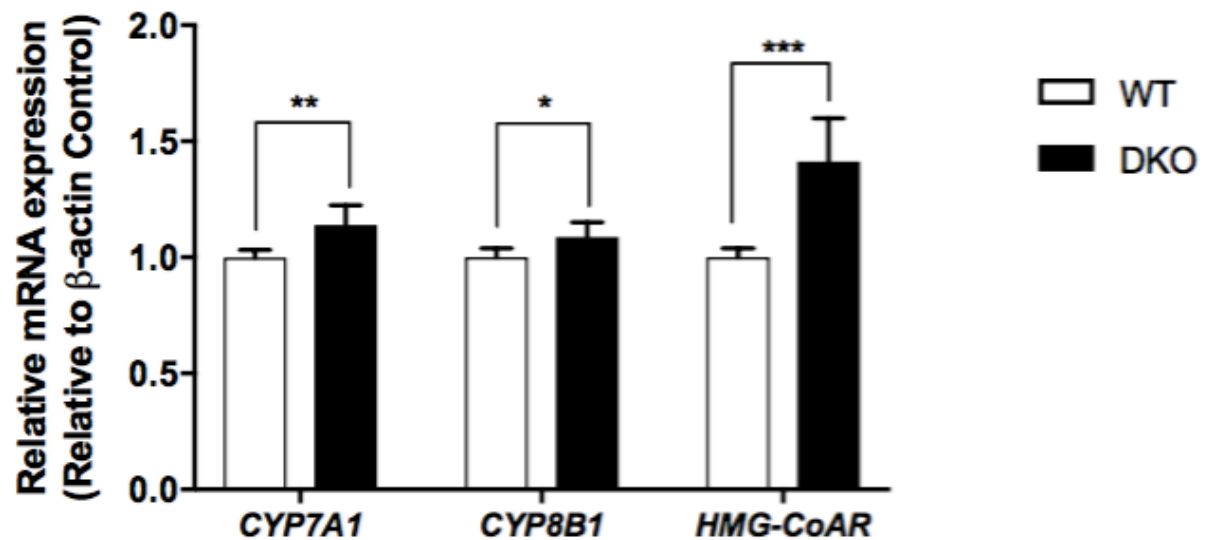


Fig. 3. The effects of BCO1/BCO2 DKO on cholesterol synthesis and bile acid synthesis biomarkers. Relative mRNA levels of cholesterol metabolism markers (*CYP7A1*, *CYP8B1*, *HMG-CoAR*) were determined by qPCR in liver samples of WT and BCO1/BCO2 DKO mice. β -actin was used as a loading control. Statistical significance was determined by the unpaired t-test. Values are mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

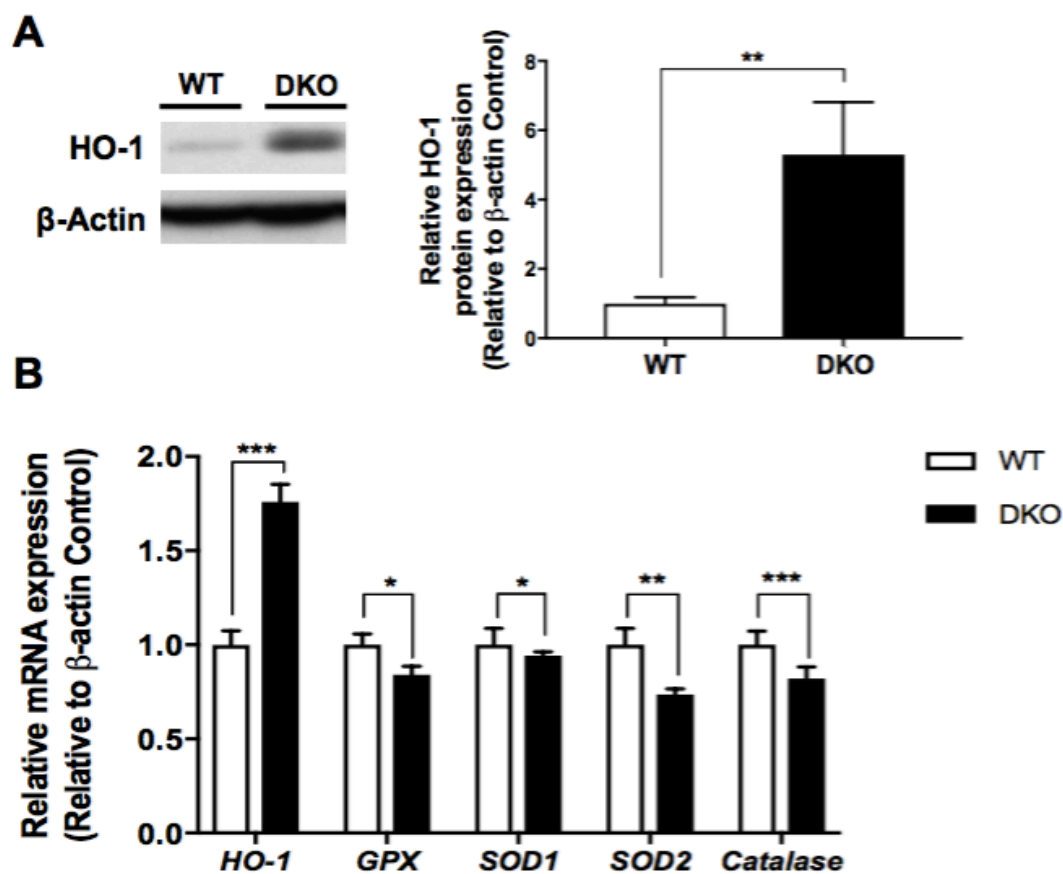


Fig. 4. The effects of BCO1/BCO2 DKO on oxidative stress biomarkers. (A) Relative protein levels of HO-1 were analyzed by western blots of liver lysates of WT and BCO1/BCO2 DKO. (B) Relative mRNA levels of anti-oxidant enzymes (HO-1, GPX, SOD1, SOD2, catalase) were detected by qPCR in liver samples of WT and BCO1/BCO2 DKO mice. Representative results are shown in the left panel. Band intensities were quantified by densitometry. β -actin was used for normalization in qPCR and western blot testing. Statistical significance was determined by the unpaired t-test. Values are mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

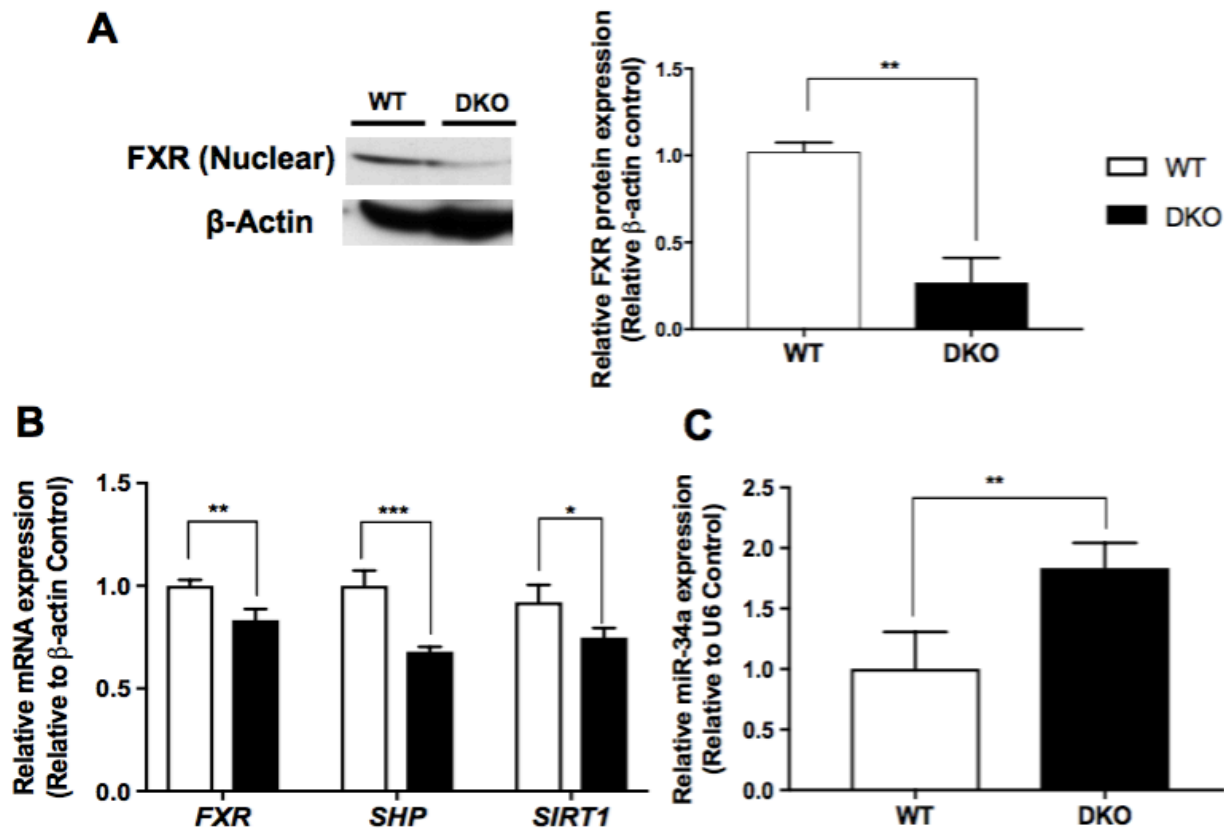


Fig. 5. The effects of BCO1/BCO2 DKO on FXR/miR-34/SIRT1 pathway. (A) Relative protein levels of FXR is analyzed by western blots of nuclear extract of livers of WT and BCO1/BCO2 DKO mice. (B) Relative mRNA levels of FXR, SHP, and SIRT1 were quantified by qPCR. Representative results are shown in the left panel. Band intensities were quantified by densitometry. β -actin was used for normalization in qPCR and western blot testing. Statistical significance was determined by the unpaired t-test. Values are mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

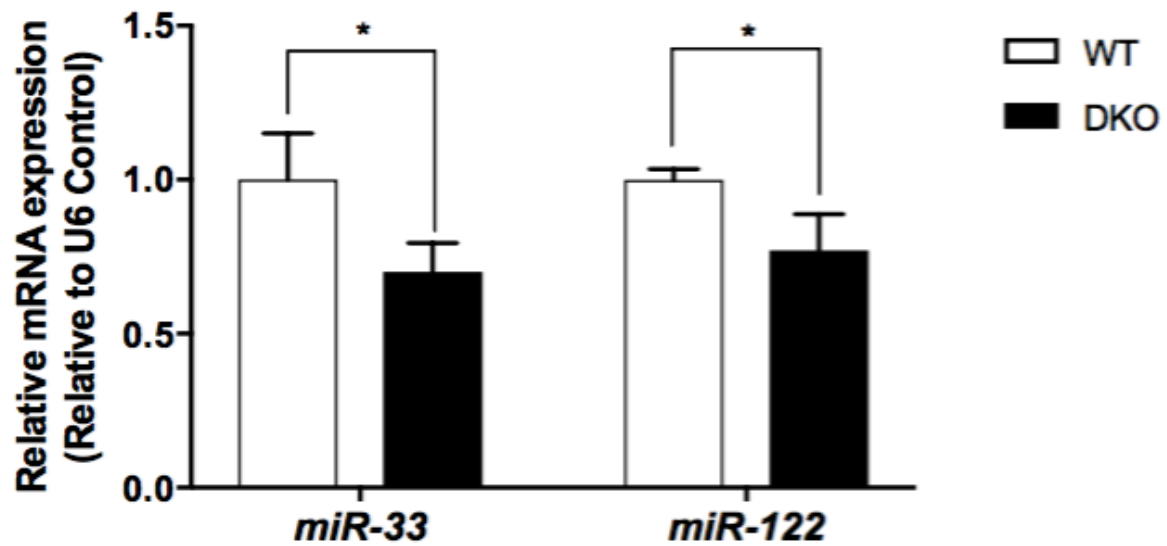


Fig. 6. The effects of BCO1/BCO2 DKO on miR-33/miR-122 axis. MiR-33 and miR-122 were determined by qPCR of liver samples from WT and BCO1/BCO2 DKO mice. Statistical significance was determined by the unpaired t-test. Values are mean $\pm$ SEM. * $P < 0.05$.

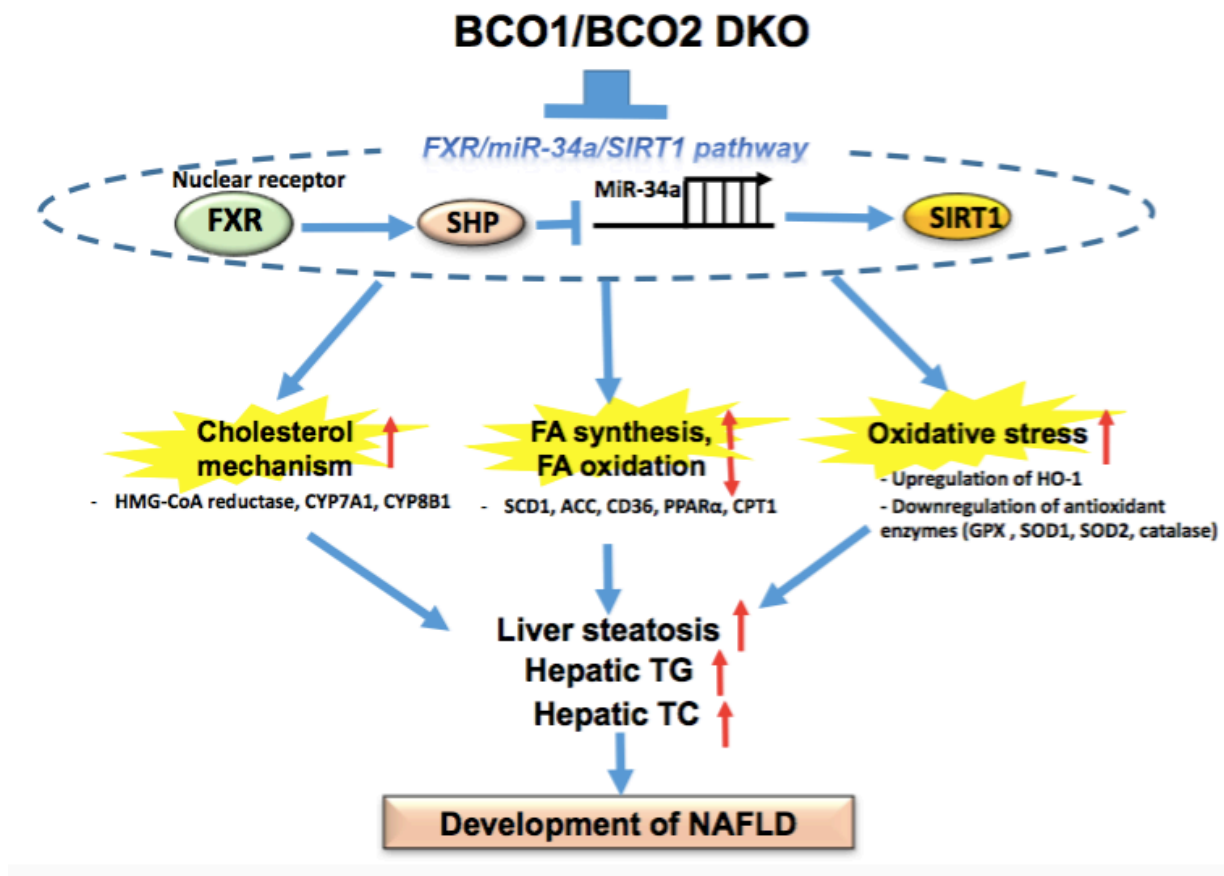


Fig. 7. The proposed mechanism of BCO1/BCO2 ablation induces NAFLD. BCO1/BCO2 DKO inactivates the FXR/miR-34a/SIRT1 pathway, leading to aberrant expression levels of miR-33/miR-122 axis; this directly and indirectly regulates various biomarkers involved in fatty acid synthesis, fatty acid β -oxidation, cholesterol metabolism and oxidative stress. These results demonstrate: 1) elevated hepatic fatty acid synthesis and reduced β -oxidation; 2) increased cholesterol synthesis and elevated cholesterol utilization; 3) increased oxidative stress and reduced anti-oxidant capacity (that may lead to increased hepatic steatosis with elevated hepatic TG and hepatic TC levels) resulting in the development of NAFLD.

Chapter 4. Project 2.

: To examine the effects of dietary BCX on the development of HRCD-induced NAFLD in mice

This study has been published

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Dietary β -Cryptoxanthin Inhibits High-Refined Carbohydrate Diet-Induced Fatty Liver via Differential Protective Mechanisms Depending on Carotenoid Cleavage Enzymes in Male Mice.

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Abstract

Background: β -Cryptoxanthin (BCX), a provitamin A carotenoid shown to protect against nonalcoholic fatty liver disease (NAFLD), can be cleaved by β -carotene-15, 15'-oxygenase (BCO1) to generate vitamin A, and by β -carotene-9', 10'-oxygenase (BCO2) to produce bioactive apo-carotenoids. BCO1/BCO2 polymorphisms have been associated with variations in plasma carotenoid levels in humans.

Objective: We investigated whether BCX feeding inhibits high refined-carbohydrate diet (HRCD)-induced NAFLD, dependent or independent of BCO1/BCO2.

Methods: Six-week-old male wild-type (WT) and BCO1^{-/-}/BCO2^{-/-} double KO (DKO) mice were randomly fed HRCD (66.5 % of energy from carbohydrate) with or without BCX (10 mg/kg diet) for 24 weeks. Pathological and biochemical variables were analyzed in the liver and mesenteric adipose tissues (MAT). Data were analyzed by 2-factor ANOVA.

Results: Compared to their respective HRCD controls, BCX reduced hepatic steatosis severity 33–43% and hepatic total cholesterol levels 43–70% in both WT and DKO mice ($P < 0.01$). Hepatic concentrations of BCX, but not retinol and retinyl palmitate, were 33-fold higher in DKO mice than in WT mice ($P < 0.001$). BCX feeding increased the hepatic fatty acid oxidation protein PPAR α and the cholesterol efflux gene *Abcg5* and suppressed the lipogenesis gene *Acc1* in the MAT of WT mice but not DKO mice ($P < 0.05$). BCX feeding decreased the hepatic lipogenesis proteins ACC and SCD1 (3- and 5-fold) and the cholesterol synthesis genes *Hmgcr* and *Hmgs1* (2.7- and 1.8-fold) and increased the cholesterol catabolism gene *Cyp7a1* (1.9-fold) in the DKO but not WT mice ($P < 0.05$). BCX feeding increased hepatic protein levels of SIRT1 (2.5-fold) and AMPK (9-fold) and

decreased hepatic FXR protein (80%) and the inflammatory cytokine gene *Il6* (6-fold) in the MAT of DKO mice but not WT mice ($P < 0.05$).

Conclusion: BCX feeding mitigates HRCD-induced NAFLD in both WT and DKO mice through different mechanisms in the liver-MAT axis, depending on the presence or absence of BCO1/BCO2.

Keywords: NAFLD, high-refined carbohydrate diet, β -cryptoxanthin, carotenoid cleavage enzymes

Introduction

Non-alcoholic fatty liver disease (NAFLD) affects 30%, 20%, and 3–10% of adult men, women, and children, respectively, in the USA, and its incidence is rapidly accelerating to cause a tidal wave of disability, illness, and death [197]. The prevalence of NAFLD is 80–90% in obese adults, 30–50% in patients with diabetes, and upwards of 40–70% among obese children [198]. Since limited treatment and pharmacological options exist for NAFLD, determining efficacy of dietary intervention in prevention of NAFLD is urgently needed.

Accumulating evidence from epidemiological and animal studies has shown that high consumption of a high-refined carbohydrate diet (HRCD), including high fructose and high sugar, is intimately associated with the development and severity of NAFLD [9, 199, 200]. This is mainly due to the deleterious effects of fructose, which can be delivered directly to the liver via the portal vein, thus contributing to liver injury [201]. We previously found that replacing high-fat diet with a HRCD (including both maltodextrin and sucrose disaccharide, which can be broken down to monosaccharides: glucose and fructose) significantly exacerbated hepatic steatosis [9]. We also observed that HRCD-fed mice exhibited more severe hepatic steatosis than high-fat diet-fed mice, which was associated with a significant upregulation of *de novo* lipogenesis (DNL) genes and proteins [10]. These findings suggest that in addition to reducing HRCD consumption, another promising strategy may be to identify bioactive dietary components that effectively target the development of HRCD-induced NAFLD.

β -Cryptoxanthin (BCX), a pro-vitamin A xanthophyll carotenoid, is found at high levels in sweet red peppers (*Capsicum annuum* L.) and its dietary product, paprika, and

at lower levels in butternut squash, pumpkins, tangerines, oranges, and papayas [23, 202]. Several epidemiological studies have shown that serum levels of BCX are inversely associated with the risk of certain types of cancers [203, 204], type 2 diabetes [205], body mass index (BMI) [206], liver disorders [207], and NAFLD [208]. Several clinical intervention trials have shown that consumption of a BCX beverage improves adipokine levels [209], BMI [210], visceral fat accumulation [211], serum cholesterol levels [212], and levels of biomarkers of oxidative stress and inflammation in NAFLD patients [213]. Previously, BCX supplementation was shown to suppress insulin resistance and NASH [27, 28] and to reduce inflammatory cytokines in adipose tissues of obese mice [214]; however, the effects of BCX feeding on HRCd-induced NAFLD development are unknown. Importantly, BCX is preferentially cleaved by β -carotene-15, 15'-oxygenase (BCO1) and β -carotene-9', 10'-oxygenase (BCO2) to produce bioactive metabolite products including vitamin A [23, 202]. However, whether the protective role of BCX is dependent on BCO1/BCO2 is unclear, and the presence of single nucleotide polymorphisms (SNPs) in BCO1/BCO2 enzymes in humans makes this a pertinent question. It has been shown that SNPs in BCO1/BCO2 enzymes are associated with carotenoid status [19], age-related eye diseases [22], and increased interleukin (IL)-18 concentrations [18].

Recently, we demonstrated that ablation of both BCO1 and BCO2 from the mice fed a standard chow diet induced NAFLD potentially through the impairment of the farnesoid X receptor (FXR) and sirtuin 1 (SIRT1) pathway [215]. FXR, a metabolic nuclear receptor, plays pivotal roles in the maintenance of bile acid homeostasis and lipid and cholesterol metabolism [216, 217]. SIRT1, a nicotinic adenosine dinucleotide (NAD⁺)-

dependent protein/histone-deacetylase, has been associated with the development of NAFLD [218]. SIRT1 is known to be a metabolic regulator of fatty acid synthesis [219], enhancer of fatty acid β -oxidation [220], and an inhibitor of inflammation [221], oxidative stress [222], and adipocyte differentiation [223]. Ablation of SIRT1 aggravated liver steatosis by facilitating the release of free fatty acids (FFAs) from mesenteric adipose tissue (MAT) [224]. The AMP-activated protein kinase (AMPK) is a master regulator of energy homeostasis and has health implications for treating insulin resistance, type 2 diabetes, and NAFLD [225]. AMPK activates SIRT1 by increasing the NAD^+/NADH ratio [226, 227] and regulating a variety of metabolic pathways including hepatic lipid accumulation [227]. Although we investigated the potential role of BCX in activating SIRT1 using a mouse model of lung cancer [52], the effects of BCX on the FXR/SIRT1/AMPK pathway remain largely obscure. Additionally, it remains unexplored whether BCX regulates the FXR/SIRT1/AMPK pathway in HRCD-induced NAFLD depending on BCO1/BCO2 activity.

In the present study, we investigated the role of BCX in the development of HRCD-induced NAFLD using BCO1/BCO2 double KO (DKO) mice lacking the carotenoid cleavage enzymes and the respective wild-type (WT) mice. We also investigated multiple molecular mechanisms and the potential involvement of the FXR/SIRT1/AMPK pathway on the BCX role in the liver-MAT axis.

Methods

2.1. Animals, diets and study design

Male wild-type (WT) and BCO1^{-/-}/BCO2^{-/-} double knockout mice (BCO1/BCO2 DKO), congenic on the C57BL/6J genetic background, were generated as previously described [215]. Since we previously observed a dramatic male predominance in the incidence of steatosis with no steatosis detected in female mice [228], only male mice were used in the present study. We previously demonstrated that WT mice fed a standard chow diet did not develop NAFLD, while DKO mice fed a standard chow diet developed very mild NAFLD (hepatic steatosis score of 1.0) [215]. Moreover, we showed that mice fed HRCD for 24 weeks showed a greater severity of NAFLD (pathologic hepatic steatosis score of 3.0) than high-fat diet-fed C57BL/6J WT mice [10]. Since our aim in the present study was to determine whether dietary BCX inhibits NAFLD in either the absence or presence of carotenoid cleavage enzymes, all WT and DKO mice were fed the same semi-purified HRCD diet used previously [10] to induce NAFLD based on assessment by histopathologic score analyses. All experimental procedures were performed in accordance with the animal protocols approved by the Tufts University Institutional Animal Care and Use Committee. After 2 weeks of acclimation, 6 week-old WT and DKO mice were randomly assigned to receive either: 1) HRCD (66.5% of calories were from carbohydrate consisting of maltodextrin and sucrose) that contained sufficient vitamin A without carotenoids, as described in our previous study [10], or 2) HRCD supplemented with BCX (daily dose of 10 mg/kg diet). All animals were fed the experimental diets ad libitum with free access to water for 24 weeks as previously described [10]. All mice were housed and maintained under environmental conditions of 22 ± 2 °C, 40 ± 5% relative humidity, and 12-h light/12-h dark cycle. During the experimental period, body weights of

all animals were measured twice weekly. At the conclusion of the dietary intervention, the animals were euthanized by terminal exsanguination (for serum) under IsothesiaTM (Henry Schein, Dublin, OH) anesthesia without overnight fasting. Liver and MAT were excised and weighed. The central left lobe of liver was collected, and fixed in 10% buffered formalin solution (Thermo Fisher Scientific, Grand Island, NY), and the remaining portions of liver were snap-frozen and stored at – 80 °C for further analysis.

2.2. BCX supplementation

The BCX dosage (10 mg/kg diet) was established from our previous work as the dose reported to reduce murine lung carcinogenesis [54]. The BCX concentrate (MJ-71-01) was provided by Dr. Vijaya Juturu (Omni Active Health Technologies Pvt. Ltd, Morristown, NJ). The BCX concentrate was extracted/purified from paprika and suspended in oil, as previously described [214]. We confirmed that the BCX concentrate contained 12.5 % BCX and no other carotenoids were detectable by our HPLC analysis. We mixed 80 mg of BCX concentrate into each 1 kg of HRCD powder diet (Bio-Serv, Flemington, NJ) under red light to obtain a final concentration of 10 mg BCX/kg diet. The diet was stored at – 20 °C. The diets were changed twice a day during feeding. We also conducted a stability test of BCX in diet and observed that BCX remained stable in the diet for 3-4 days. The dose selected for use in this study is equivalent to a daily human consumption of 0.87 mg, which is comparable to consuming 85.0–113 g of sweet red pepper daily [54], and thus this dose of BCX is considered physiologically relevant.

2.3. Liver histopathology evaluation

Five-micrometer sections of formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin to determine the liver steatosis score as described previously [215]. Sections were examined under a light microscope by two independent investigators who were blinded to the treatment groups, as described previously [215].

2.4. Reverse transcription-PCR and quantitative real-time PCR (qRT-PCR)

Total RNA extraction from liver and MAT samples and subsequent qRT-PCR were performed as previously described [215]. Quantitative PCR was performed on an Applied Biosystems 7000 machine with FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, IN). Selected primers are described in Supplemental Table 1 and β -actin was used as an internal control.

2.5. MicroRNA quantification by real-time qRT-PCR analysis

Total RNA extraction and subsequent cDNA synthesis for microRNA-34a analysis were performed as previously described [215]. Quantitative PCR was performed on an Applied Biosystems 7000 machine with FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, IN). U6 snRNA was used as an internal control.

2.6. Western blot analysis

Western blot analyses were performed in the liver tissues from all animals as previously described [215]. The following antibodies were used: SIRT1 (Santa Cruz, TX); FXR

(GeneTex, Irvine, CA), PPARA (Abcam, Cambridge, MA); SCD1, ACC, total AMPK (Cell Signaling, Beverly, MA), and β -actin (Sigma-Aldrich, Milan, Italy). The densities of specific relative bands were quantified by densitometric scanning using a model GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA); β -actin was used to confirm equal loading.

2.7. Hepatic triglyceride (TG), total cholesterol (TC), and plasma free fatty acid (FFA) analysis Hepatic TG, TC, and plasma FFA levels were measured using a commercial TG colorimetric assay kit (Cayman, Ann Arbor, Michigan), TC assay kit (Cell Biolabs Inc, San Diego, CA), and FFA quantification assay kit (Abcam, Cambridge, UK), respectively. All procedures were performed according to the protocol obtained from the manufacturer.

2.8. High-performance liquid chromatography (HPLC) analysis

Liver samples for HPLC analysis were prepared as previously described [215]. The levels of retinol, retinyl palmitate, and BCX were analyzed in the liver tissue using gradient reversed phase HPLC consisting of a Waters 2996 photodiode array detector (Waters, Milford, MA), a Waters 2695 separation module and a reverse-phase C18 column (4.6 $\times$ 250 mm, 5 μ m) with a flow rate of 1.00 mL/min. Retinol, retinyl palmitate, and BCX concentrations were calculated by determining the peak areas in the chromatograms calibrated against known amounts of standard. All procedures were conducted under red light.

2.9. Statistical analysis

All statistical analyses were performed using GraphPad-Prism 7.0 software (GraphPad Software). Results are presented as the mean $\pm$ standard error of the mean (SEM) or medians. Data were tested for normal distribution by Shapiro-Wilk normality test. Two-way analysis of variance (ANOVA) was performed to assess BCX, strain, and BCX-strain interactions among groups. One-way ANOVA followed by Tukey's post-hoc analysis was conducted when significant effects were detected using a two-way ANOVA. Nonparametric Kruskal-Wallis test with Benjamini-Hochberg FDR for multiple testing corrections was used to compare hepatic scores, and correlations were tested by Spearman's test. To determine differences in variances for ANOVAs, Bartlett's test was performed. For hepatic BCX concentrations, comparisons between the two genotypes were analyzed by Student's *t*-test. Differences among groups were regarded statistically different when $P < 0.05$.

Results

BCX suppresses hepatic lipid accumulation in both WT and DKO mice

Mean final body weight (g) and liver weight/body weight did not differ significantly among all groups (Table 1). MAT weight/body weight were significantly lower in the HRCD and HRCD+BCX groups of the DKO mice than in the WT-HRCD group ($P < 0.05$), but there were no significant differences between HRCD and HRCD+BCX group irrespective of

genotype (Table 1). Histological analyses demonstrated that HRCD induced pronounced hepatic steatosis in both WT and DKO mice (score 3.0 and 3.5, respectively). However, BCX intervention significantly suppressed hepatic steatosis in both genotypes (score 2.0) when compared to that in their HRCD littermates ($P < 0.01$ for both; Table 1). Histopathological examination revealed that, irrespective of genotype, the presence of large and small fat droplets was markedly reduced by BCX in mice compared to that in their HRCD littermates (Fig. 1). BCX feeding significantly reduced hepatic TC content by 43% and 70%, respectively, in the WT and DKO mice when compared to that in their HRCD littermates ($P < 0.001$ for both; Table 1). Furthermore, BCX feeding significantly decreased hepatic TG (36%) in the DKO mice when compared to that in their HRCD littermates ($P < 0.05$; Table 1). In both genotypes, hepatic TG and TC were correlated with the hepatic steatosis score ($\rho = 0.36, 0.72$; $P = 0.0193, < 0.0001$, respectively). Together, these results indicate that BCX exerted ameliorative effects on HRCD-induced hepatic steatosis in both WT and DKO mice.

Retinol, retinyl palmitate, and BCX levels

Hepatic retinol and retinyl palmitate levels did not significantly differ among all experimental groups (Table 1). BCX was not detected in the HRCD groups without BCX feeding in WT and DKO mice (Table 1). BCX levels in the liver were significantly higher (33-fold) in the DKO-HRCD+BCX group than in the WT-HRCD+BCX group ($P < 0.001$; Table 1). We did not detect any other carotenoids or apo-carotenoids (such as β -apo-10'-carotenal or 3-hydroxy- β -apo-10'-carotenal) in the livers of any groups.

Differential actions of BCX on lipid metabolism in WT and DKO mice

The imbalance between lipogenesis and fatty acid β -oxidation can result in hepatic lipid accumulation in the liver. In DKO mice, but not in WT mice, BCX significantly lowered the mRNA levels of lipogenesis markers including fatty acid synthase (*Fas*) and acetyl-CoA carboxylase 2 (*Acc2*) by 40% and 42%, respectively ($P < 0.01$ and $P < 0.01$, respectively), compared to those in the HRCD group (Fig. 2A and C). Moreover, the protein levels of lipogenesis markers including ACC and stearoyl-CoA desaturase-1 (SCD1) were significantly downregulated (3-fold and 5-fold, respectively) by BCX feeding in DKO mice, but not in WT mice, as compared to levels in their HRCD littermates ($P < 0.05$ for both; Fig. 2F). Interestingly, *Fas* mRNA and SCD1 protein expressions were significantly higher (30% and 6-fold, respectively) in the DKO-HRCD group, as compared with those in the WT-HRCD group ($P < 0.05$ for both; Fig. 2A and F). In WT mice, but not in DKO mice, BCX significantly increased gene expression levels of fatty acid β -oxidation markers, including peroxisome proliferator-activated receptor- α (*Ppara*) and acyl-CoA oxidase 1 (*Acox1*), (26% for both; $P < 0.05$) compared to those in the HRCD group (Fig. 2D, E). PPARA protein levels were also significantly induced (2.5-fold) by BCX feeding in WT mice, but not in DKO mice, compared to those in their HRCD littermates ($P < 0.05$; Fig. 2F). These results suggest that BCX impacts lipid metabolism differently depending on the presence or absence of carotenoid cleavage enzymes. BCX suppressed lipogenesis in DKO mice and increased fatty acid β -oxidation in WT mice, which can contribute to the mitigation of liver steatosis in both genotypes.

Differential actions of BCX on cholesterol metabolism in WT and DKO mice

Hepatic cholesterol homeostasis is maintained by a coordinated regulation of cholesterol biosynthesis and uptake, cholesterol conversion to bile acids, and cholesterol efflux. In DKO mice, but not in WT mice, BCX significantly increased (1.9-fold) the mRNA expression levels of hepatic cholesterol 7 α -hydroxylase (*Cyp7a1*), a rate-limiting enzyme for bile acid synthesis, when compared to the levels in HRCD mice (Fig. 3A). Notably, BCX significantly reduced mRNA levels of 3-hydroxy-3-methyl-glutaryl-coenzymeA reductase (*Hmgcr*) and HMG-CoA synthase 1 (*Hmgs1*) in DKO mice by 2.7-fold and 1.8-fold, respectively, when compared to the levels in their HRCD littermates ($P < 0.001$ and $P < 0.05$, respectively; Fig. 3C and D). In WT mice, but not in DKO mice, BCX significantly up-regulated (2.5-fold) the mRNA levels of the cholesterol transporter, ATP-binding cassette transporter 5 (*Abcg5*), when compared to the levels in their HRCD littermates ($P < 0.05$; Fig. 3E). The gene expression of LDL-receptor (*Ldlr*), cholesterol uptake marker, was not significantly different among groups (data not shown). HRCD-fed DKO mice had significant increases in cholesterol synthesis biomarkers including Sterol regulatory element-binding protein 2 (*Srebf2*) and *Hmgcr* compared to HRCD-fed WT mice (1.5- and 3.0-fold respectively) ($P < 0.001$ and $P < 0.0001$, respectively; Fig. 3B and C). These data suggest that BCX contributes to reducing hepatic TC levels by promoting cholesterol catabolism and reducing cholesterol biosynthesis in DKO mice while increasing cholesterol efflux in WT mice.

Differential actions of BCX on nuclear receptors in WT and DKO mice

In DKO mice, BCX significantly suppressed both gene and protein expression of FXR (69% and 80% respectively) when compared to the expression in their HRCD littermates ($P <$

0.01 for both; Fig. 4A and B). In WT mice, however, BCX resulted in significant elevation in protein levels of FXR (2.0-fold) and mRNA levels of its downstream target, small heterodimer partner (*Shp*) (50%), compared to their expression in their HRCD littermates ($P < 0.001$ and $P < 0.05$, respectively; Fig. 4B and C). These data suggest that BCX regulates the nuclear receptors differently, depending on BCO1/BCO2.

Intact BCX activates the SIRT1/AMPK pathway in the livers of DKO mice

Although no significant differences in *Sirt1* mRNA expression were observed among all groups (Fig. 5A), BCX significantly up-regulated SIRT1 protein levels (2.5-fold) in DKO mice, but not in WT mice, when compared to the levels in the HRCD group ($P < 0.05$; Fig. 5B). Moreover, BCX significantly induced AMPK protein levels (9-fold) in DKO mice, but not in WT mice, when compared to the levels in their HRCD littermates ($P < 0.05$; Fig. 5B). The levels of microRNA-34a (miR34a), which is a direct inhibitor of SIRT1, were not significantly different among groups (Fig. 5C). These data suggest that intact BCX ameliorated hepatic steatosis potentially by up-regulating the SIRT1/AMPK pathway, especially in DKO mice.

Intact BCX increases fatty acid β -oxidation and suppresses IL-6 and IL-4 potentially by elevating Sirt1 levels in the MAT of DKO mice

In WT mice, but not in DKO mice, BCX significantly reduced *Acc1* mRNA levels (70%) when compared to the levels in the HRCD group ($P < 0.05$; Fig. 7A). Of note, BCX significantly induced *Acox1* mRNA levels (2.6-fold) in DKO mice, but not in WT mice, when compared to the HRCD group ($P < 0.05$; Fig. 7B). Interestingly, pro-inflammatory

cytokine, *Il6* mRNA levels were significantly induced by HRCD and attenuated by BCX (6-fold) in DKO mice ($P < 0.01$; Fig. 7C). Moreover, an anti-inflammatory cytokine, *Il4* mRNA levels were significantly increased by HRCD and inhibited by BCX (5.5-fold) in DKO mice ($P < 0.01$; Fig. 7D). Notably, we observed that BCX significantly increased mRNA levels of *Sirt1* approximately by 2.5-fold in DKO mice when compared to the levels in the HRCD group ($P < 0.001$; Fig. 7E). These data suggest that BCX enhanced fatty acid β -oxidation and suppressed the *Il6* and *Il4*, potentially by elevating *Sirt1* levels in DKO mice.

Discussion

In the present study, we provided evidence that dietary BCX effectively prevents the development of HRCD-induced NAFLD in both BCO1/BCO2 DKO mice and the corresponding WT control mice. Notably, our data suggest that the mechanisms involved are different in WT and DKO mice, indicating that the BCX molecule, when not cleaved, exerts independent functions. The BCX-induced mitigation of hepatic steatosis in the absence of BCO1/BCO2 suggests that intact dietary BCX plays a role in the prevention of NAFLD. There were no significant differences in the hepatic levels of hepatic retinol or retinyl palmitate among the groups, implying that intact BCX has unique roles in the prevention of HRCD-induced NAFLD, without generating vitamin A. Importantly, we observed that the protective effects of BCX against NAFLD are achieved through different molecular mechanisms in the liver-MAT axis and depend on carotenoid cleavage enzymes.

We demonstrated that BCX effectively suppressed hepatic TG in DKO mice, partly through the suppression of *de novo* lipogenesis (DNL). While a large proportion of hepatic TG is derived from plasma FFAs [229], DNL is the major contributor to the synthesis of TG in NAFLD [230]. Thus, the BCX-mediated reduction in TG levels in DKO mice potentially resulted from the suppression of hepatic DNL, which was associated with an increase in SIRT1 and AMPK protein levels by BCX feeding in DKO mice. This increase in SIRT1 and AMPK protein levels has been reported to result in the inhibition of FAS and ACC expression and the prevention of lipid accumulation in the liver [227], indicating that intact BCX-mediated activation of the SIRT1/AMPK pathway possibly contributes to the inhibition of lipogenesis markers in the livers of DKO mice. Unlike in DKO mice, the induction of SIRT1 and anti-lipogenic effects of BCX was not observed in WT mice. Additionally, in WT mice, the levels of the fatty acid β -oxidation markers PPAR α and ACOX1 were increased, possibly due to the upregulation of FXR and the subsequent induction of hepatic nuclear SHP [231]. In line with our results, several studies have reported that FXR/SHP activation improves hyperlipidemia [159] and liver steatosis [232, 233]. Overall, our results suggest that the effects of BCX on lipogenesis and fatty acid β -oxidation depend on the intact BCX molecule or its cleavage derivatives, respectively.

Animal and clinical studies have shown that BCX effectively reduces plasma and hepatic cholesterol levels [28, 212, 214, 234]. Interestingly, we observed that BCX mitigated hepatic TC accumulation in both DKO and WT mice. It is possible that a combination of increased cholesterol bioconversion into bile acids and decreased cholesterol biosynthesis by BCX contributes to the mitigation of hepatic TC accumulation in DKO mice. This notion was supported by our observation that BCX markedly increased

Cyp7a1 mRNA expression in DKO mice, possibly resulting from FXR suppression, which reportedly reduces hypercholesterolemia by inducing CYP7A1 [235-237]. BCX has also been reported to induce the expression of CYP27A1, an enzyme that is involved in the conversion of excess cholesterol into 27-hydroxycholesterol [238]. It is thus conceivable that intact BCX suppressed FXR and subsequently activated CYP7A1, thereby increasing hepatic TC catabolism and consequently reducing hepatic TC in DKO mice. Given that AMPK has been shown to directly inhibit FXR transcriptional activity [239], we speculate that the inhibition of FXR by BCX in DKO mice might be due to AMPK activation. In addition to the beneficial effects of FXR activation, accumulating evidence also suggests that FXR inactivation is beneficial for ameliorating dyslipidemia, especially hypercholesterolemia [236, 237]. Our results align with those of the recent study by Kim *et al.* showing that deletion of FXR improved hepatic lipid accumulation by up-regulating *Cyp7a1* gene in aged mice [240]. Moreover, we demonstrated that intact BCX effectively inactivates the expression of the cholesterol biosynthesis markers (including *Hmgcr* and *Hmgs1*) in DKO mice [241]. This probably occurs through the up-regulation of SIRT1 and AMPK induced by BCX feeding. Inhibition of SIRT1 and AMPK in NAFLD is reportedly associated with high cholesterol synthesis via downregulation of the inhibition of HMGCR phosphorylation [241, 242]. Unlike in DKO mice, in WT mice, the cholesterol efflux was a major contributor to the BCX-mediated decrease in hepatic TC levels, which may have been affected by upregulation of FXR and SHP expression [243]. Together, our results suggest that BCX regulates cholesterol metabolism through differential regulation of the FXR/SIRT1/AMPK pathway in the liver, depending on BCO1/BCO2. However, the

mechanisms underlying FXR/SIRT1/AMPK regulation by BCX, depending on the presence or absence of BCO1/BCO2, remain elusive and require further research.

In the current study, the MAT/body weight ratio was slightly but significantly lower in the DKO-HRCD mice than in the WT-HRCD mice. The hepatic steatosis score, hepatic steatosis incidence, hepatic TG, and hepatic TC were marginally but not significantly higher in DKO-HRCD than in WT-HRCD mice. These metabolic perturbations may result from the deficiency of the BCO2 enzyme as reported by Ford *et al.* who observed that BCO2 KO mice exhibited significantly lower visceral adiposity and hepatic enlargement with lipid accumulation, suggesting that BCO2 may have specific roles in the regulation of lipid metabolic homeostasis between liver and adipose tissue [45]. Thus, it is plausible that the reduction of MAT in DKO-HRCD mice, relative to WT-HRCD group, occurred because of the ablation of BCO2. Consistent with this hypothesis, we found that the lipogenesis marker (*Acc1*) was significantly lowered in the MAT, while the lipogenesis markers (*Fas* and *SCD1*) and the cholesterol synthesis markers (*Srebf2* and *Hmgcr*) were significantly higher in the liver in the DKO-HRCD group than in the WT-HRCD group.

MAT is regarded as potentially harmful because it contains higher levels of the pro-inflammatory cytokine IL-6 than other adipose tissues [244], which plays a crucial role in NAFLD progression [245]. The higher levels of IL-6 in MAT might directly or indirectly contribute to hepatic damage similar to insulin resistance [246] and to the severity of NAFLD [247]; therefore, reducing IL-6 in MAT is a potential strategy for preventing NAFLD. Our results show that an elevation of IL-6 was present in the MAT of DKO-HRCD mice and was suppressed by BCX. Moreover, in the present study, we observed high levels of the anti-inflammatory cytokine IL-4 in the MAT of the DKO-HRCD group which were

significantly reduced by BCX feeding. Of note, IL-4 has been demonstrated to contribute to the obesity in humans [248, 249], and play a role in the improvement of adipose tissue dysfunction by countering pro-inflammatory functions [250]. Our results suggest that both pro-inflammatory response and anti-inflammatory response were elevated in the MAT of DKO-HRCD mice and were suppressed by BCX. It has been suggested that fatty acid β -oxidation reduces the expression of IL-6 in adipocytes [251]. Similarly, our results suggest that BCX may induce the fatty acid β -oxidation marker (*Acox1*) in the MAT, which might lead to the reduction of *Il6* in DKO mice. The BCX-mitigated *Il6* in the MAT might be ascribed to the up-regulation of *Sirt1*, which is associated with the inhibition of inflammatory cytokines such as TNF- α and IL-6 in adipose tissues [221] and the regulation of PGC-1 α -regulated enzymes involved in fatty acid β -oxidation, including ACOX1 [252]. Similarly, BCX administration has been reported to increase the fatty acid β -oxidation marker PPARA in visceral fat [214]. In WT mice, we demonstrated that BCX significantly reduced the levels of the lipogenesis marker *Acc1* in the MAT. Taken together, our findings imply that BCX differentially regulates lipid metabolism and pro- and anti-inflammatory cytokine levels in the MAT, depending on the presence of BCO1/BCO2.

The dose of BCX (10 mg/kg diet) used in this study was equivalent to the consumption of 0.87 mg/day (which would be easily achievable by consuming 85–113 g (or 1-2 medium-sized) of sweet red peppers [52, 54]. This dose is within the physiological range based on the BCX levels in WT-HRCD+BCX group, which had the carotenoid cleavage enzymes. The mean BCX levels in the livers of WT-HRCD+BCX mice (0.79 nmol/g tissue) were comparable to the levels of BCX in human liver tissues (0.1–3.5

nmol/g tissue) [253]. Our results are consistent with those reported from a clinical study in which the consumption of a beverage containing 3 mg/day of BCX by patients improved the metabolic risk parameters in NAFLD patients [213]. We found that BCX levels in the DKO- HRCD+BCX mice were significantly higher than those in the WT-HRCD+BCX mice. Considering that DKO mice do not have carotenoid cleavage enzymes, it was expected that DKO mice significantly accumulate BCX in the liver. Other studies have reported that the accumulation of carotenoids in mice with either one of the carotenoid cleavage enzymes removed resulted in deleterious effects including oxidative stress [16] and fat accumulation [15]. In contrast, we observed beneficial effects of BCX accumulation on HRCD-induced NAFLD in DKO mice; this may be possibly due to the lower dose of BCX utilized in our study (10 mg/kg diet) compared to that used by others (50 mg~1g/kg diet) [15, 16].

Since the BCX levels in DKO-HRCD+BCX mice were significantly (approximately 33-fold) higher than those in WT-HRCD+BCX mice, we cannot exclude the possibility that differences in the accumulation of BCX between the DKO and WT mice play a role in terms of the protective effect of BCX. Different amounts of BCX may also diversely impact specific molecular pathways. Additionally, we did not detect any other carotenoids and BCX metabolites aside from vitamin A in both WT and DKO mice. Therefore, our data support that the inhibition of HRCD-induced NAFLD may be mainly due to BCX.

In conclusion, the findings from this study suggest that BCX exerts protective effects against HRCD-induced NAFLD by regulating multiple molecular pathways in the liver-MAT axis. The molecular mechanisms, however, differed in terms of BCX regulation, which depended on BCO1/BCO2, suggesting that intact BCX has a unique biological

function in the prevention of HRCD-induced NAFLD. Together, our results strongly suggest that the consumption of abundant fruits and vegetables enriched in BCX may be necessary to lower the risk of NAFLD associated with the high consumption of refined carbohydrate/sugar diets, regardless of the presence of BCO1/BCO2 polymorphisms in humans.

Table 2. Primary outcomes of WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX

Experimental group	WT		BCO1/BCO2 DKO		<i>P</i> values for two-way ANOVA		
	HRCD	HRCD+BCX	HRCD	HRCD+BCX	BCX effect	Strain effect	BCX-strain interaction
Number of mice	15	15	15	15			
Final body weight ¹ (g)	46.0 ± 0.91	46.6 ± 1.07	46.2 ± 0.84	46.8 ± 1.00	0.52	0.82	0.96
Liver weight ¹ (g)	2.89 ± 0.27	2.34 ± 0.16	2.73 ± 0.17	2.91 ± 0.21	0.38	0.34	0.08
Liver weight/Body weight ¹ , %	6.23 ± 0.48	5.06 ± 0.34	5.85 ± 0.31	6.14 ± 0.37	0.35	0.25	0.06
Mesenteric fat weight ¹ (g)	0.98 ± 0.09	0.88 ± 0.04	0.78 ± 0.17	0.77 ± 0.04	0.36	<0.05	0.46
Mesenteric fat weight/Body weight ¹ , %	2.11 ± 0.18 ^a	1.92 ± 0.08 ^{ab}	1.69 ± 0.08 ^b	1.65 ± 0.09 ^b	0.25	<0.01	0.41
Hepatic steatosis ² incidence, % of mice	93% (14/15)	93% (14/15)	100% (15/15)	80% (12/15)	-	-	-
Hepatic steatosis score, median ¹ (range)	3.0 (0-4) ^a	2.0 (0-3) ^b	3.5 (1-4) ^a	2.0 (0-3) ^b	-	-	-
Hepatic TG level ¹ (mg/g)	19.0 ± 1.26 ^{ab}	15.6 ± 1.16 ^{bc}	20.8 ± 1.72 ^a	13.4 ± 1.19 ^c	<0.05	0.36	0.23
Hepatic TC level ¹ (mg/g)	97.5 ± 11.5 ^a	55.7 ± 6.26 ^b	120 ± 8.45 ^a	51.0 ± 7.08 ^b	<0.001	0.38	0.12
Plasma FFA ¹ (μM)	159 ± 2.23 ^a	162 ± 4.87 ^a	144 ± 6.46 ^{ab}	127 ± 4.86 ^b	0.18	<0.001	0.06
Hepatic retinol ¹ (nmol/g)	6.61 ± 1.22	6.08 ± 1.24	5.87 ± 1.33	5.25 ± 0.79	0.65	0.53	0.97
Hepatic retinyl palmitate ¹ (mmol/g)	0.28 ± 0.08	0.40 ± 0.09	0.28 ± 0.08	0.34 ± 0.24	0.29	0.73	0.56
Hepatic BCX ¹ (nmol/g)	N/D	0.50 ± 0.04	N/D	16.7 ± 3.84 [*]	<0.001	<0.001	<0.001

Abbreviations: BCO1/BCO2 double knock-out (DKO); FFA, free fatty acids; HRCD, high-refined carbohydrate diet; HRCD+BCX, high-refined carbohydrate diet supplemented with β -cryptoxanthin; N/D, not detectable; TC, total cholesterol; TG, triglycerides; WT, wild-type.

¹Values are presented as means $\pm$ SEM, n = 10–15 (final body weight, liver weight, mesenteric fat weight, TG, TC, and FFA) or 8 (hepatic retinol, retinyl palmitate, and BCX) or medians (ranges), n = 15 (hepatic steatosis score). Means without a common letter differ ($P < 0.05$). *Different from WT-HRCD+BCX group, $P < 0.05$.

²Hepatic steatosis is defined as intrahepatic lipid accumulation exceeding 5% of the liver weight.

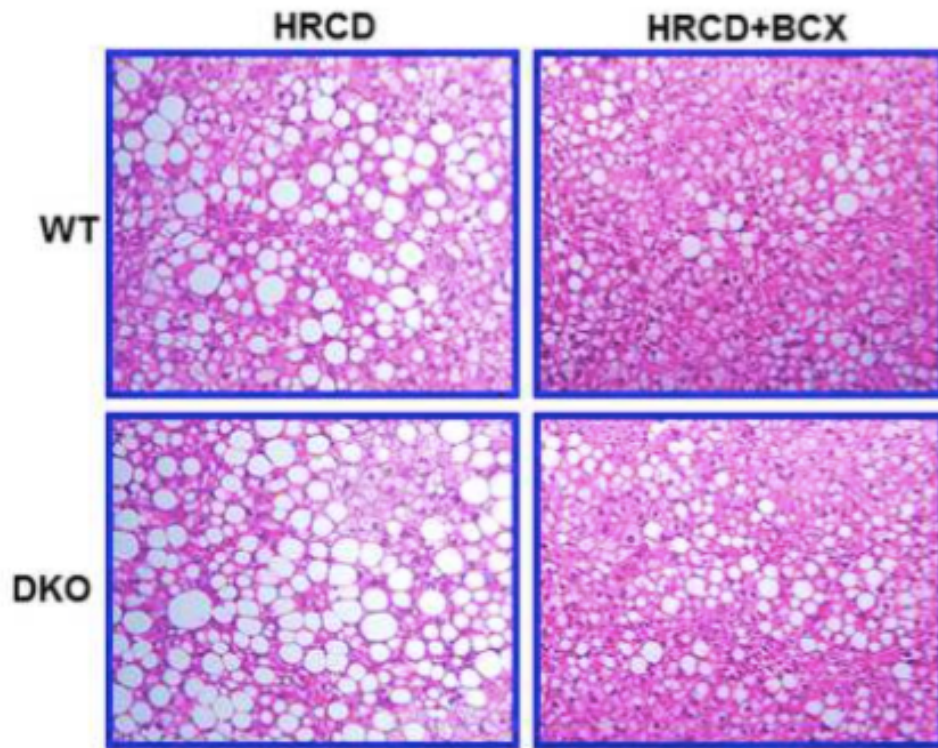


Fig. 1. Histopathological analysis of the liver of the WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Hematoxylin and eosin-stained slides were imaged microscopically and photographed at 100 $\times$. BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; WT, wild-type.

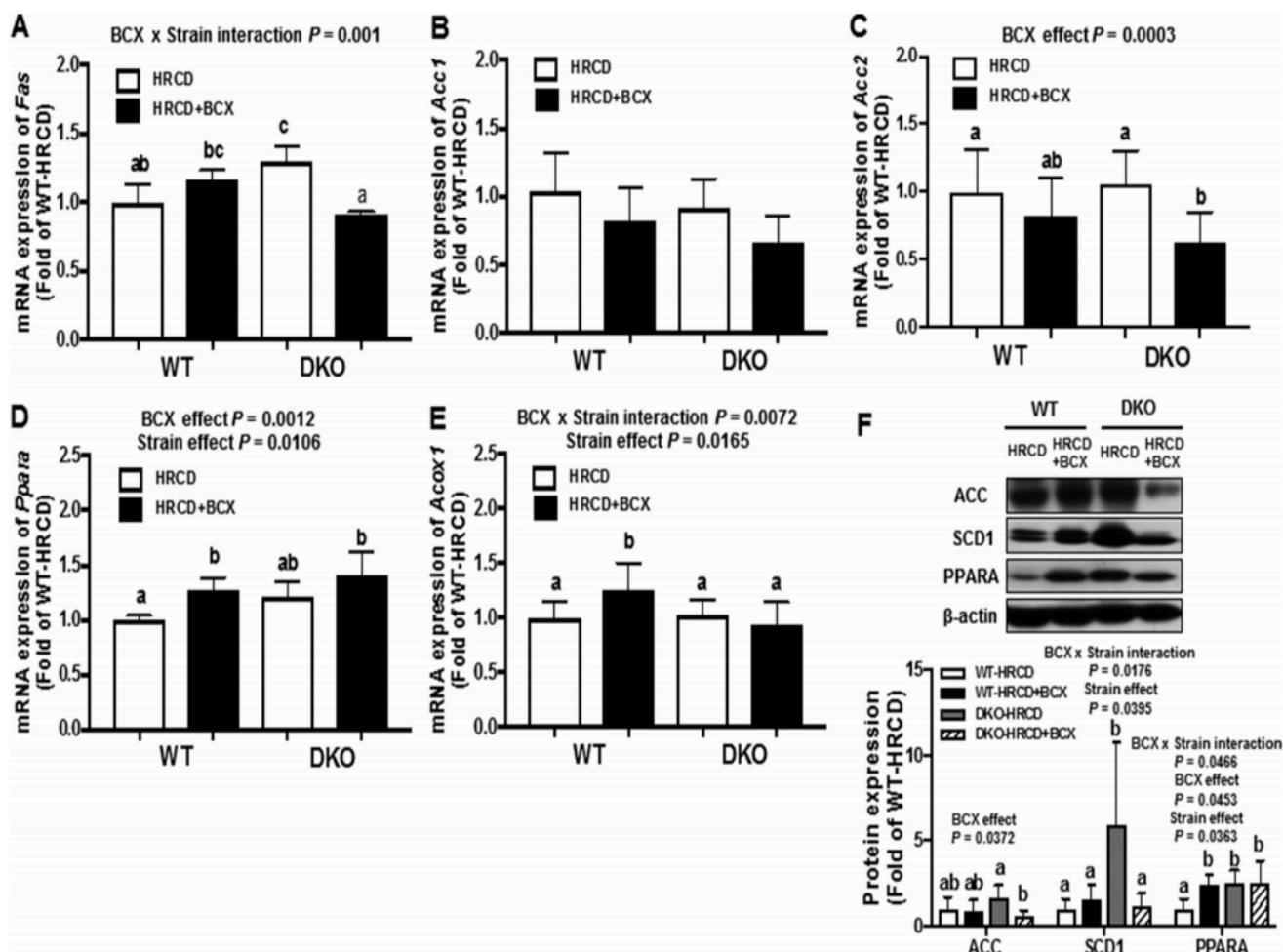


Fig. 2. Effects of BCX on hepatic lipid metabolism markers in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in the mRNA expression of fatty acid synthesis markers (A) *Fas*, (B) *Acc1*, and (C) *Acc2* and fatty acid β -oxidation markers (D) *Ppara*, (E) *Acox1*, and (F) ACC, SCD1, and PPARA proteins quantified via real-time PCR or western blot in the livers of WT mice and DKO mice fed HRCD with or without BCX. β -actin was used as a loading control. Values are means $\pm$ SEM, $n = 10$ – 12 except panel F where $n = 5$ or 6 . Means without a common letter differ, $P < 0.05$. *Acc*, acyl CoA carboxylase; *Acox1*, acyl CoA oxidase 1; BCX, β -cryptoxanthin; DKO, double knockout; *Fas*, fatty acyl synthase; HRCD, high-refined carbohydrate diet; *Ppara*, peroxisome proliferator activated receptor- α ; SEM, standard error of the mean; Stearoyl-CoA desaturase-1, SCD1; WT, wild-type.

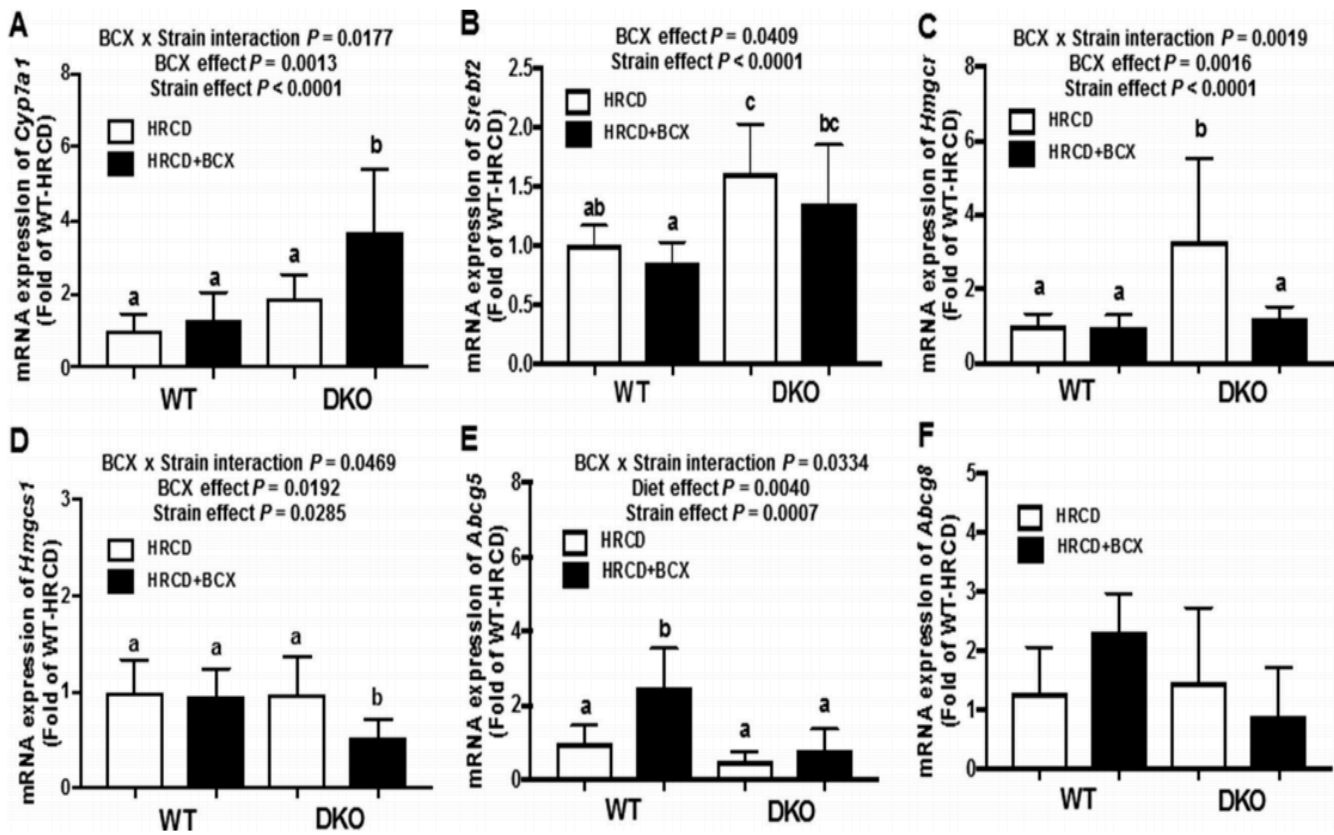


Fig. 3. Effects of BCX on hepatic cholesterol metabolism markers in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in the mRNA expression of bile-acid synthesis markers (A) *Cyp7a1*, cholesterol synthesis markers, (B) *Srebf2*, (C) *Hmgcr*, and (C) *Hmgcs1* and cholesterol efflux markers, (D) *Abcg5*, and (E) *Abcg8* quantified via real-time PCR in the livers of WT mice and DKO mice fed HRCD with or without BCX. β -actin was used as a loading control. Values are means $\pm$ SEM, $n = 10$ – 12 . Means without a common letter differ, $P < 0.05$. *Abcg5*, ATP-binding cassette transporter5; *Abcg8*, ATP-binding cassette transporter8; BCX, β -cryptoxanthin; *Cyp7a1*, cholesterol 7 α -hydroxylase; DKO, double knockout; *Hmgcr*, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, *Hmgcs1*, HMG-CoA synthase 1; HRCD, high-refined carbohydrate diet; *Srebf2*, sterol regulatory element-binding protein-2; SEM, standard error of the mean; WT, wild-type.

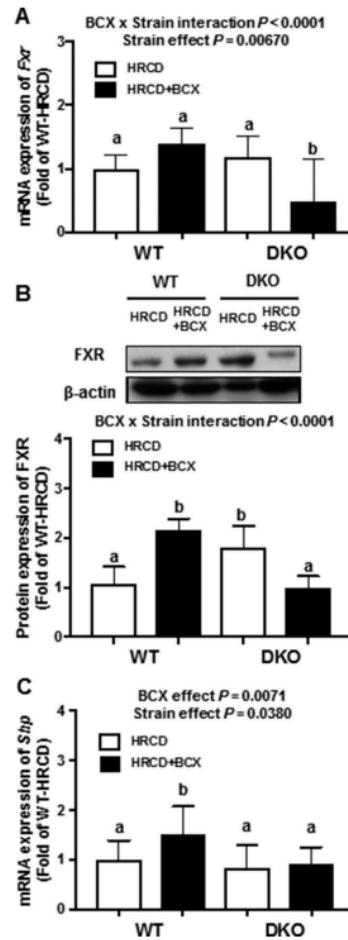


Fig. 4. Effects of BCX on nuclear receptor FXR and SHP in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in the mRNA and protein expression of nuclear receptors. (A) *Fxr* mRNA (n = 10–12), (B) FXR protein (n = 6), and (C) *Shp* mRNA (n = 10–12) quantified via real-time PCR and western blot in the livers of WT mice and DKO mice fed with HRCD supplemented with or without BCX. β -actin was used as a loading control. Values are means $\pm$ SEM, n = 10–12 except panel B where n = 6. Means without a common letter differ, $P < 0.05$. BCX, β -cryptoxanthin; DKO, double knockout; *Fxr*, farnesoid X receptor; HRCD, high-refined carbohydrate diet; SEM, standard error of the mean; *Shp*, small heterodimer partner; WT, wild-type.

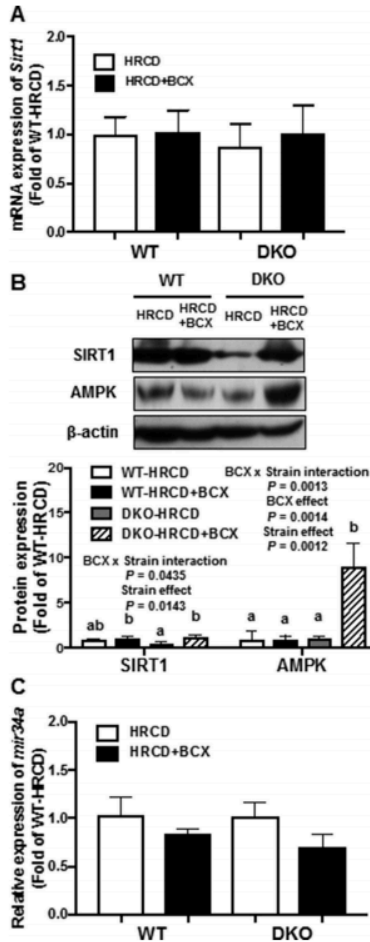


Fig. 5. Effects of BCX on the SIRT1/AMPK pathway in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in mRNA levels of (A) *Sirt1* and protein levels of (B) SIRT1, AMPK, and (C) miR34a quantified via real-time PCR and western blot in the livers of WT mice and DKO mice fed with HRCD supplemented with or without BCX. β -actin was used as loading control in real-time PCR and western blot testing. U6 snRNA was used as a loading control for miR34a. Values are means $\pm$ SEM, $n = 10$ – 12 except panel B and C where $n = 6$. Means without a common letter differ, $P < 0.05$. BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; miR, microRNA; SEM, standard error of the mean; SIRT1, sirtuin1; WT, wild-type.

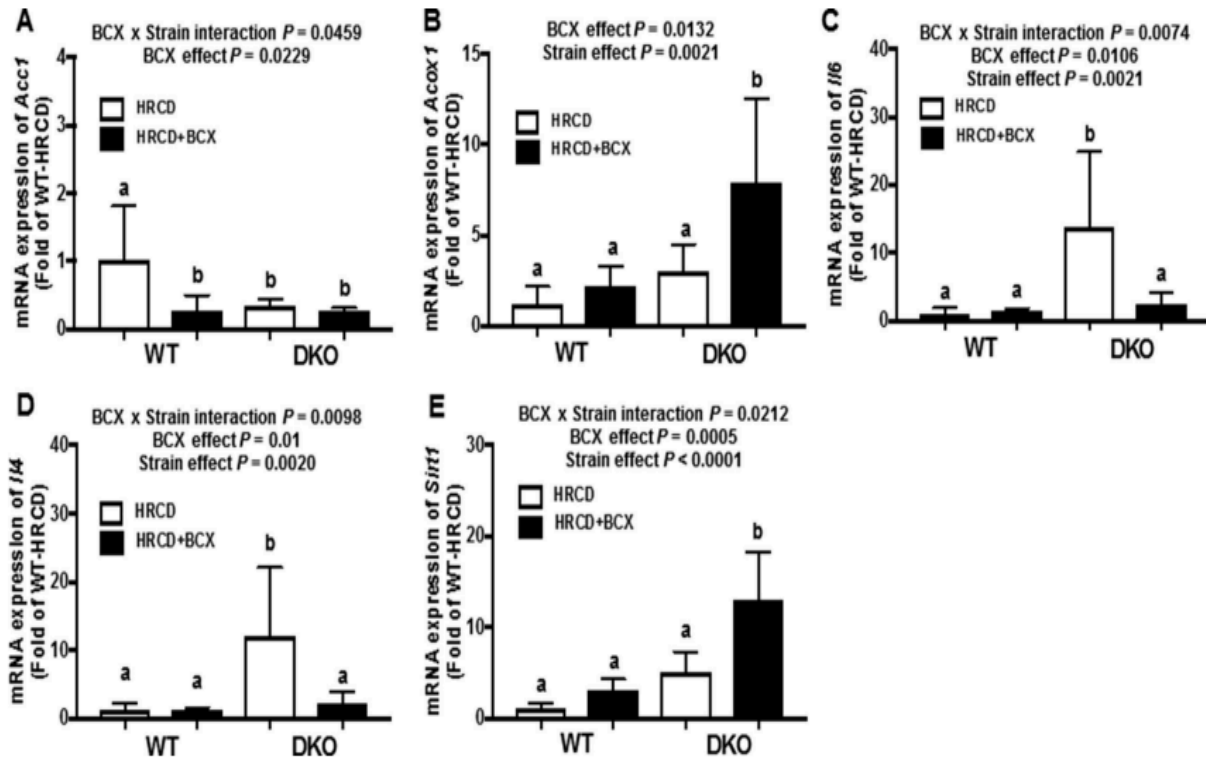


Fig. 6. Effects of BCX on mesenteric lipid metabolism, inflammation, and SIRT1 in MAT of WT mice and BCO1/BCO2 DKO mice fed HRCD with or without BCX supplementation. Graphical representation of fold changes in the mRNA expression of (A) *Sirt1*, (B) *Acc1*, (C) *Acox1*, (D) *Il6*, and (E) *Il4* quantified via real-time PCR in the livers of WT mice and DKO mice fed HRCD with or without BCX. β -actin was used as loading control. Values are means $\pm$ SEM, $n = 10$ – 12 . Means without a common letter differ, $P < 0.05$. *Acc1*, acyl CoA carboxylase1; *Acox1*, acyl CoA oxidase1; BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; *Il6*, interleukin-6; *Il4*, interleukine-4; SEM, standard error of the mean; WT, wild-type.

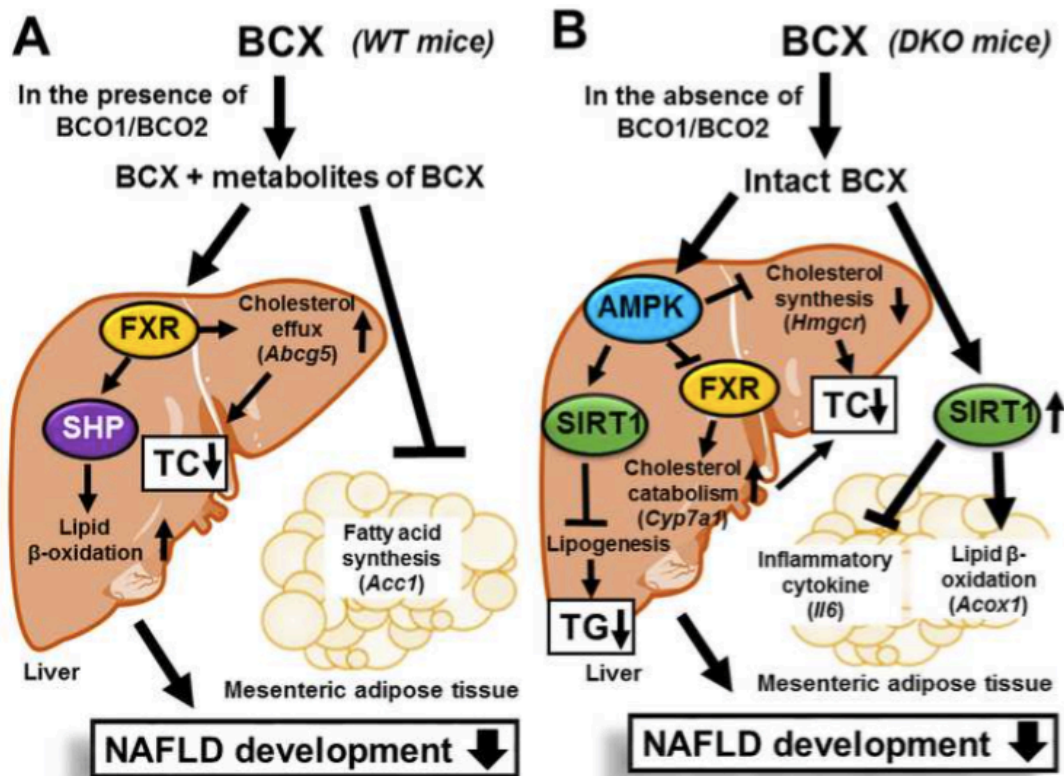


Fig. 7. The proposed mechanism underlying the prevention of HRCD-mediated NAFLD by BCX in WT mice and BCO1/BCO2 DKO mice fed HRCD with or without BCX. (A) In WT mice, BCX up-regulates FXR and subsequently activates SHP, thus elevating fatty acid β -oxidation and cholesterol efflux in the liver. BCX also inhibits lipogenesis in the MAT. All these factors potentially contribute to the prevention of HRCD-induced NAFLD. (B) In DKO mice, BCX up-regulates AMPK and subsequently activates SIRT1, thereby inhibiting lipogenesis and cholesterol biosynthesis in the liver. Intact BCX down-regulates FXR possibly by AMPK activation, thereby up-regulating CYP7A1, potentially elevating cholesterol catabolism in the liver. BCX also up-regulates SIRT1, thus inhibiting IL-6 and increasing fatty acid β -oxidation in the MAT. All these factors potentially contribute to the prevention of HRCD-induced NAFLD. BCX, β -cryptoxanthin; CYP7A1, cholesterol 7 α -hydroxylase; DKO, double knockout; FXR, farnesoid X receptor; HRCD, high-refined carbohydrate diet; IL-6, interleukin-6; MAT, mesenteric adipose tissue; NAFLD, non-alcoholic fatty liver disease; SHP, small heterodimer partner; SIRT1, sirtuin1; WT, wild-type.

Chapter 5. Project 3.

: To observe the effects of dietary BCX on DEN-initiated, HRCD-promoted hepatocellular carcinoma in mice

Abstract

Scope: β -Cryptoxanthin (BCX), a provitamin A carotenoid, can be cleaved by both beta-carotene 15,15'-oxygenase (BCO1) and beta-carotene 9',10'-oxygenase (BCO2) generating biological active vitamin A and apocarotenoids. The aim of this study was to determine whether BCX feeding could inhibit diethylnitrosamine-initiated, HRCD-promoted HCC development, dependent or independent of BCO1/BCO2 activity.

Methods and results: Male wild-type (WT) and BCO1^{-/-}/BCO2^{-/-} double knock-out (DKO) mice were fed HRCD (66.5 % of energy from carbohydrate) with or without BCX for 24 weeks. BCX feeding increased hepatic vitamin A levels in WT mice, but not in DKO mice that showed a significant accumulation of hepatic BCX. Compared to their respective HRCD littermates, both WT and DKO fed BCX had significantly lower HCC multiplicity (58-60%), average tumor size (21-24%), and total tumor volume (51-58%), and the steatosis scores. The chemopreventive effects of BCX were associated with increased p53 protein acetylation and gluconeogenesis markers (PEPCK, G6Pase) and decreased protein levels of a glycolysis marker (LDHA) and of the hypoxia-inducible factor-1 α and its downstream targets, matrix metalloproteinase 2/9 in tumors.

Conclusions: This study suggests that BCX feeding may alleviate HRCD-promoted HCC progression by modulating the acetylation of p53, hypoxic tumor microenvironment and glucose metabolism, independent of BCO1 and BCO2.

1. Introduction

Hepatocellular carcinoma (HCC), the most prevalent subtype of primary liver cancer, is the third most common cause of cancer death worldwide, and its incidence is increasing worldwide [254]. While mortality for all common cancers continues to decline in the United States, it has been suggested that deaths related to liver cancer will increase by 2030 [255]. Numerous risk factors, including hepatitis B and C infections, as well as obesity and non-alcoholic fatty liver disease (NAFLD), are associated with the occurrence of HCC [256, 257].

Studies have demonstrated that high consumption of a high-refined carbohydrate diet (HRCD) containing excessive amounts of refined sugar is associated with the development of metabolic syndrome-associated HCC [8, 258]. We showed that replacing a high fat diet with a HRCD significantly exacerbates hepatic steatosis and induces comparable HCC development in mice, despite the fact that the HRCD-fed mice weighed less than high fat diet-fed mice [10]. These data suggest that HRCD-induced metabolic syndrome and inflammation, independent of obesity, may contribute to HCC development in the rodent model. Further, it has been suggested that reducing the intake of dietary refined carbohydrates could lower inflammation and tumor cell growth [259]. These data support the association between HRCD-induced metabolic syndrome and the risk of developing HCC. Since the consumption of refined carbohydrates and the incidence of HCC continue to increase in the general population [260], the underlying mechanisms for the association of HRCD and HCC need to be explored.

Cancer cells tend to have an altered energy metabolism, known as the Warburg effect, that is characterized by a higher glucose uptake and glycolysis rather than

oxidative phosphorylation [125]. The Warburg effect has been widely examined in human cancers, including HCC [126], and is closely associated with the aggressiveness and high proliferation of the cancer [127, 128]. Lactate dehydrogenase (LDHA) has been reported to be a critical enzyme involved in glycolysis and proliferation in HCC [129]. LDHA inhibition has been shown to mitigate HCC progression [261]. In addition, a recent study demonstrated that enhancing gluconeogenesis in malignant hepatocytes inhibited glycolysis and led to suppression of HCC progression [262], suggesting that targeting glucose metabolism may potentially be a novel therapeutic strategy for suppressing HCC progression.

The tumor microenvironment is characterized by hypoxia that is thought to contribute to the development and progression of NAFLD-driven HCC [263]. Hepatic lipid accumulation has been reported to induce hypoxia in the hepatic microenvironment predisposing the liver to hepatic injuries and HCC [264]. Hypoxia inducible factor-1 α (HIF-1 α) is stabilized under an hypoxic tumor microenvironment resulting in the up-regulation of LDHA and matrix metalloproteinases (MMPs) that increases glycolysis and cell migration/invasion, respectively, contributing to HCC progression [265]. Of note, p53, the most important tumor suppressor, functions as a transcription factor regulating the expression of LDHA, HIF-1 α , cell cycle, and apoptosis targets [266, 267]. Emerging studies have revealed that acetylation of p53, the active form of p53, could be a key modulator of the anti-cancer effects of p53 by stabilizing its activity [268, 269].

β -Cryptoxanthin (BCX) is a natural pro-vitamin A xanthophyll carotenoid found in sweet red peppers (*Capsicum annuum* L.), paprika, butternut squash, and pumpkins [23, 202]. A recent clinical intervention study also reported that cumulative liver cancer

incidence was significantly lower among hepatitis patients with cirrhosis who were given a BCX-enriched Mandarin orange juice in addition to a carotenoid mixture capsule-treated group, compared to a control group [36]. To date, whether BCX has its impact on HRCD-promoted HCC development/progression and if so, what is the underlying mechanisms, has not been investigated. Of note, BCX can be cleaved by β -carotene-15, 15'-oxygenase (BCO1) and β -carotene-9', 10'-oxygenase (BCO2) to generate bioactive metabolites that include vitamin A [23, 202]. We have previously demonstrated that BCX supplementation can reduce the multiplicity of tobacco carcinogen-induced lung tumors, without altering levels of vitamin A (retinol and retinyl ester) in both serum and lung tissues [54]. These data suggest that the observed beneficial effects of BCX against cancer development may be independent of its provitamin A activity. However, it is unclear whether BCX can inhibit HRCD-promoted hepatic carcinogenesis independent of carotenoid cleavage enzymes, and their products such as retinol and other biological metabolites. Single nucleotide polymorphisms (SNPs) of BCO1/BCO2 genes have been reported in humans and related to a variety of conditions [19, 22]. Indeed, we have previously reported that BCO1^{-/-}/BCO2^{-/-} double knockout mice developed liver steatosis and have significantly higher hepatic and serum levels of triglyceride and total cholesterol compared to wild type animals [215]. Furthermore, several recent studies suggested that BCO1 and BCO2 could serve as tumor suppressor genes [49-51]. Since observation of these biological functions of BCO1 or BCO2 has thus far been limited to the *in vitro* and xenograft studies, more relevant evidence involving use of a murine knockout model is necessary for better understanding the roles of BCO1 and BCO2 in cancer development.

To address the issues mentioned above, in the current study we investigated whether dietary BCX could inhibit the development/progression of HRCD-promoted HCC and affect glucose metabolism and hypoxic tumor microenvironment. We also explored whether the protective effects of BCX feeding were independent of BCO1/BCO2 by comparing the results from wild-type (WT) versus BCO1/BCO2 double knock-out (DKO) mice.

2. Experimental section

2.1. Animals, diets, and study design

Male BCO1^{-/-}/BCO2^{-/-} double knockout mice (BCO1/BCO2 DKO) on a C57BL/6J genetic background and wild-type (WT) littermates were studied as previously described [215]. Only male mice were used in the current study because in humans the liver tumor incidence is higher in males than in females [255]. All animal experimental procedures were conducted in accordance with animal protocols approved by the Tufts University Institutional Animal Care and Use Committee. At 2 weeks of age, WT and DKO mice were injected intraperitoneally with 25 mg/kg body weight diethylnitrosamine (DEN), a liver-specific carcinogen, as previously reported [270]. At 6 weeks of age, WT and DKO mice were randomized by weight and were fed semi-purified diets containing either: 1) HRCD (66.5% of total daily caloric intake as carbohydrates consisting of sucrose and maltodextrin) with sufficient vitamin A and without carotenoids, or 2) HRCD supplemented with BCX (10 mg/kg diet), as previously described [10]. During the 24-week of dietary intervention, all animals were individually housed in individual cages in an AAALAC - accredited animal facility in a controlled environment (12 h light/12 h dark cycle) with free access to diet and water [10]. Body weight was recorded twice weekly. At the end of the

feeding period, mice were euthanized by terminal exsanguination under IsothesiaTM (Henry Schein, Dublin, OH) anesthesia.

2.2. BCX supplementation

The BCX concentrate (12.5% BCX, Omni Active Health Technologies Pvt. Ltd, Morristown, NJ) was mixed directly into the HRCD semi-purified powder diet (Bio-Serv, Flemington, NJ) at 10 mg BCX/kg diet, a dose found to suppress lung carcinogenesis in mice), as previously described [54, 271]. This BCX dose is equivalent to 0.87 mg/day for human consumption, which can be achieved by consuming 85–113 g of sweet red pepper daily [54] and thus regarded to be physiologically relevant. Since BCX in the diet is stable for 3-4 days at –20°C, as previously reported [271], the diets were prepared twice per week during the experimental period.

2.3. Liver tumor quantification and tumor volume calculation

The tumors in liver were counted and excised. Two blinded investigators measured the tumor diameters with a caliper, and tumor volume was calculated using the formula $V = \frac{4}{3}\pi r^3$, where $r = \text{diameter}/2$. The central left lobe of each liver was collected, and fixed in 10% buffered formalin solution (Thermo Fisher Scientific, Grand Island, NY) for histological analysis. The remaining liver samples were snap-frozen in liquid nitrogen and stored at –80°C.

2.3. Liver Histopathology

Five-micrometer sections of formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E) to evaluate the liver steatosis score in non-tumor areas [215]. To confirm tumor identity as HCC, two investigators who were blinded to the treatment groups evaluated and scored samples based on the criteria previously described [270].

2.4. Reverse transcription-PCR and quantitative real-time PCR (qRT-PCR)

Total RNA extraction from liver tumor tissue was performed using Trizol reagent (Invitrogen, Waltham, MA), and subsequent quantitative real-time PCR (qRT-PCR) was carried out as previously described [215]. QTT-PCR was conducted on an Applied Biosystems 7000 Sequence Detection System with FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, IN). Relative mRNA was calculated using the Ct method using β -actin as the internal control.

2.6. Western blot analysis

Frozen liver tumor samples were homogenized as previously described [215]. The following antibodies were used: Bax (Santa Cruz, TX); PEPCCK and CyclinD1 (Abcam, Cambridge, MA); LDHA, Bcl-xl, acetylated-p53 and total p53 (Cell Signaling, Beverly, MA), G6Pase and HIF-1 α (Nuvus Biologicals, Littleton, CO) and β -actin (Sigma-Aldrich, Milan, Italy). Quantification was performed using GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA). Relative protein expression was calculated using β -actin as a loading

control.

2.8. High-performance liquid chromatography (HPLC) analysis

Liver tissue samples were homogenized for HPLC analysis of BCX, retinol and retinyl palmitate, as previously described [215]. HPLC was performed using gradient reversed phase HPLC consisting of a Waters 2996 photodiode array detector (Waters, Milford, MA), a Waters 2695 separation module, and a reverse-phase C18 column (4.6 × 250 mm, 5 µm) with a flow rate of 1.00 mL/min. The identification and quantification of retinol, retinyl palmitate, and BCX were conducted by determining the peak areas on the chromatograms for each standard. All procedures were performed under red light.

2.9. Statistical analyses

All statistical analyses were carried out using GraphPad-Prism 7.0 software (GraphPad Software). Results are expressed as the mean ± standard error of the mean (SEM). In the analysis of the primary outcomes (tumor number, average tumor size, and total tumor volume), two-way analysis of variance (ANOVA) was utilized to assess BCX effect, genotype effect, and BCX-genotype interaction. Hepatic steatosis scores were compared using a nonparametric Kruskal-Wallis test. Liver tumor incidence was compared using a Chi-square test. Correlations were assessed by Spearman's test. A student's t-test was used when comparing two groups. Statistical significance is designated as $P < 0.05$.

3. Results

3.1. Body weight, liver weight, and analysis of hepatic retinol, retinyl palmitate, and BCX

There were no significant differences in mean final body weight (g), liver weight (g), and liver weight/body weight (%), among groups (Fig. 1A). While both BCO1/BCO2 DKO mice and WT mice fed diets with the same dose of BCX, the DKO mice exhibited a significant yellow coloration of the liver and adipose tissue due to BCX accumulation, which was not observed in the WT mice (Fig. 1B). Hepatic levels of retinol and retinyl palmitate were higher in the WT mice fed with BCX, compared with HRCD littermates (by 2.6– and 3.5–fold, respectively; $P = 0.06$ and $P < 0.01$, respectively) (Fig. 1C). There was no difference in hepatic levels of retinol and retinyl palmitate among DKO mice-fed with BCX or HRCD control, and WT mice fed HRCD control diet. Levels of BCX in the livers of DKO mice were significantly higher compared to the WT mice fed the same diet (by 6.6–fold; $P < 0.05$) (Fig. 1C). Other carotenoids (β -carotene, lutein, lycopene, and apo-carotenoids such as 3-hydroxy- β -apo-10'-carotenal or β -apo-10'-carotenal) were not detectable in the livers of any dietary group.

3.2. Dietary BCX inhibits HRCD-promoted HCC tumor progression in both WT and DKO mice

BCX-fed mice showed a reduced number of liver surface tumors (Fig. 2A) and a smaller tumor size as visualized by H&E staining, in both WT and DKO mice, compared to their HRCD littermates (Fig. 2B). BCX-fed mice had reduced tumor incidence by 22% in WT, although the difference was not significantly different as compared to the HRCD group (Fig. 2C). However, BCX-fed mice had significantly reduced tumor incidence by 23% in

DKO mice, as compared to the HRCD littermates ($P < 0.05$; Fig. 2C). Moreover, BCX-fed mice had significantly lower tumor number (60 % for WT; 58 % for DKO; $P < 0.05$ and $P < 0.01$, respectively) (Fig. 2C), smaller average tumor size (24 % for WT; 21 % for DKO; $P < 0.01$ and $P < 0.001$, respectively) (Fig. 2C), and total tumor volume (58 % for WT; 51 % for DKO; $P < 0.01$ for all) (Fig. 2C), irrespective of genotype (WT vs DKO), compared to their respective HRCD littermates. Moreover, livers from WT and DKO mice fed HRCD supplemented with BCX had lower lipid droplet accumulation as seen with H&E staining (Fig. 2D), and exhibited significantly lower hepatic steatosis scores (scores of 2.0 and 2.5, respectively; $P < 0.05$ and $P < 0.01$, respectively) (Fig. 2D), compared to their HRCD littermates. The hepatic steatosis score was associated with average tumor size ($\rho = 0.35$, $P < 0.05$). Together, these results suggest that dietary BCX exerts similar and beneficial effects against HRCD-promoted HCC progression in both WT and DKO mice.

3.3. Dietary BCX inhibits glycolysis but enhances gluconeogenesis in liver tumors of WT and DKO mice

To evaluate whether dietary BCX feeding affects glucose metabolism of hepatic tumors, we analyzed glycolysis and gluconeogenesis enzymes in the liver tumors of WT and DKO mice. There were no significant differences in mRNA levels of glycolysis markers, including glucose transporter 1 (GLUT1), hexokinase1 (HK1), fructose 1,6-bisphosphatase (FBPase), and pyruvate dehydrogenase (PDK1), between groups (Fig. 3A). Importantly, the protein levels of LDHA were significantly reduced in hepatic tumors of BCX-fed WT and DKO mice, compared to the HRCD littermates (by 44% for both; $P < 0.05$ for both) (Fig. 3B). Furthermore, the mRNA and protein levels of a gluconeogenesis

marker, phosphoenolpyruvate carboxykinase (PEPCK) were significantly induced in BCX-fed WT mice, but not in DKO mice, compared to those in the HRCD group (2.4– and 1.8–fold, respectively, $P < 0.05$ and $P < 0.001$, respectively) (Fig. 3A and C). Additionally, the mRNA and protein levels of the gluconeogenesis marker, glucose 6-phosphatase (G6Pase), were significantly elevated in the liver tumors of BCX-fed DKO mice, compared to the levels in the HRCD littermates (by 2.6– and 1.5–fold, respectively; $P < 0.05$ and $P < 0.001$, respectively) (Fig. 3D). Protein levels of G6Pase, but not mRNA levels, were markedly up-regulated by BCX feeding in the liver tumors of WT mice (by 1.6–fold; $P = 0.06$) (Fig. 3D). Together, these results suggest that BCX feeding inhibits glycolysis, while enhancing gluconeogenesis in hepatic tumors, which may contribute to suppression of HRCD-promoted HCC in both WT and DKO mice.

3.4. Dietary BCX suppresses HIF-1 α and its downstream targets, MMP2/9 in liver tumors of WT and DKO mice

To explore the effects of dietary BCX on the hypoxic tumor microenvironment, protein levels of HIF-1 α and its downstream targets, MMP2 and MMP9, were analyzed in liver tumors of WT and DKO mice. BCX feeding significantly down-regulated HIF-1 α in both WT and DKO mice, compared to those in the HRCD group (by 2–fold for both; $P < 0.01$ for both) (Fig. 4A). BCX feeding resulted in a 58% and 57% decrease in MMP2 protein in WT and DKO mice, respectively, compared to the levels of their respective HRCD littermates ($P < 0.01$ for both) (Fig. 4B). Moreover, BCX feeding led to a 43% and 25% decreased expression of MMP9 proteins, respectively, compared to those of HRCD littermates ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 4B). These data suggest that BCX

attenuates the hypoxic tumor microenvironment by reducing HIF-1 α , MMP2, and MMP9 in tumors, thereby inhibiting HRCD-promoted HCC progression in WT and DKO mice.

3.5. Dietary BCX induces apoptosis and inhibits proliferation in the liver tumors of WT and DKO mice

To evaluate the effects of BCX on apoptosis and cell proliferation, apoptosis markers (Bcl-xl and Bax) and CyclinD1, a cell cycle regulator, were analyzed in liver tumors of WT and DKO mice. In WT, but not in DKO mice, BCX feeding significantly down-regulated protein levels of Bcl-xl, an anti-apoptosis marker, compared with levels in HRCD littermates (by 2.4-fold; $P < 0.05$) (Fig. 5A). Additionally, BCX feeding significantly up-regulated protein expression of BAX, a pro-apoptotic marker in both WT and DKO mice, compared to those in HRCD littermates (by 80% and 45%, respectively; $P < 0.01$ and $P < 0.05$, respectively) (Fig. 5A). We further calculated the Bax/Bcl-xl ratio to assess the apoptosis potential of liver tumor cells of WT and DKO mice. BCX feeding was found to up-regulate the BAX/Bcl-xl ratio in WT mice, with a similar trend in DKO mice, compared to levels in HRCD littermates (by 60% and 32%, respectively; $P < 0.05$ and $P = 0.06$, respectively) (Fig. 5B). Moreover, BCX feeding markedly attenuated cyclinD1 protein levels in WT, with a similar trend in DKO mice, compared to those in the HRCD littermates (by 58% and 37%, respectively; $P < 0.05$ and $P = 0.06$, respectively) (Fig. 5C). These data indicate that BCX attenuates HRCD-promoted HCC progression by inducing apoptosis and inhibiting cyclin D1 in the liver tumors of both WT and DKO mice.

3.6. Dietary BCX increases p53 acetylation of liver tumors of WT and DKO mice

We assessed whether BCX feeding regulates p53 by post-translational modification by the western blot analysis of acetylation of p53 at lysine 382 and total p53. Interestingly, the p53 acetylation at lysine 382 was significantly induced by BCX feeding in both WT and DKO mice, compared with their HRCD littermates (by 10.8– and 3.6–fold, respectively; $P < 0.05$) (Fig. 6) while total p53 protein levels were significantly reduced by BCX feeding in both WT and DKO mice compared with their HRCD littermates (by 1.8– and 2.3–fold, respectively; $P < 0.05$ and $P < 0.001$, respectively) (Fig. 6). Therefore, the ratio of acetylated-p53-to-total p53 was significantly higher in the HRCD supplemented with BCX group, compared with the HRCD group, for both WT and DKO mice (21.3– and 9.3–fold, respectively; $P < 0.001$ and $P < 0.05$, respectively) (Fig. 6). These data suggest that BCX feeding increases p53 acetylation, thus inducing activation of p53-mediated pathways in liver tumors of WT and DKO mice, which may ultimately lead to suppression of HRCD-promoted HCC progression.

4. Discussion

The current study has provided the first evidence that dietary BCX feeding may have protective effects against HRCD-promoted HCC progression regardless of the presence of (WT mice) or absence (DKO mice) of BCO1/BCO2. BCX treatment reduced liver tumor size, tumor volume, and tumor number in both WT and DKO mice, suggesting that the protective effect of BCX feeding is independent of cleavage by BCO1 and BCO2. These effects of BCX were associated with the BCX's ability to target 1) glucose metabolism, 2) the hypoxic tumor microenvironment, and 3) proliferation and apoptosis,

potentially through up-regulating p53 acetylation in the liver tumors of both WT and DKO mice (Fig. 7).

In the current investigation, we found that BCX feeding resulted in a significant increase in acetylated-p53, and the acetylated-p53/p53 ratio in the liver tumors of both WT and DKO mice. Accumulating evidence has shown that increased acetylation of p53 through the inhibition of histone deacetylases induces suppression of many cancers including HCC [272]. Interestingly, BCX feeding resulted in up-regulation of p53 acetylation which may have led to the suppression of LDHA protein levels and the increase in mRNA and protein levels of gluconeogenesis enzymes in the liver tumors of WT and DKO mice. Increasing evidence indicates that the tumor-suppressive functions of p53 are associated with its regulation of glucose metabolism in cancer [273, 274]. Our findings are in agreement with previous studies reporting that p53 negatively regulates LDHA expression [274] and positively regulates PEPCK1 and G6Pase [275]. HCC tumors exhibit a high rate of glycolysis and a low rate of gluconeogenesis [262]. In HCC, the loss of gluconeogenesis is a key event required for the maintenance of a high glycolysis rate [262]. Moreover, switching from glycolysis to gluconeogenesis was found to inhibit HCC progression [262]. It is conceivable that BCX feeding may cause a shift in glucose metabolism from glycolysis to gluconeogenesis in liver tumors, which may contribute to the suppression of HCC progression in both WT and DKO mice. The underlying mechanisms by which BCX regulates acetylated-p53 still require further investigation; however, based on the current results we speculate that the metabolic shift in glucose metabolism in the liver tumors resulting from BCX feeding may be due to up-regulation of acetylated-p53, the active form of p53. Together, our findings indicate that the up-

regulation of acetylated-p53 by BCX feeding may have a beneficial effect on glucose metabolism in liver tumors, thereby contributing to the suppression of HCC progression.

In the current study, we observed a strong positive correlation between the hepatic steatosis score and the average size of the liver tumors. Given that the HIF-1 α signaling pathway is a key driver in the development of NAFLD-driven HCC, we speculate that HIF-1 α protein suppression by BCX feeding may have resulted in favorable primary outcomes of NAFLD-driven HCC in both WT and DKO mice (Fig. 2). HIF-1 α has been shown to be regulated by p53 [276]. P53 inhibits HIF-1 α activity, mostly under the extreme hypoxic tumor environment [277]. Kaluzová et al. showed that DNA damage was a fundamental factor for the p53 stabilization necessary to degrade HIF-1 α [278]. Since DEN injection could induce DNA damage in this mouse model [279], it is conceivable that the BCX-mediated acetylation of p53 inhibited HIF-1 α in a DNA damage-inducing environment. Moreover, the HIF-1 α signaling pathway plays a vital role in the HCC progression, since it functions as a transcription factor that modulates genes involved in glucose metabolism, cell invasion and migration, and angiogenesis [265]. Particularly, HIF-1 α is known to regulate LDHA expression, thereby ensuring a low dependence on oxygen in a hypoxic tumor microenvironment [280]. Therefore, we hypothesize that BCX-mediated HIF-1 α inhibition may result in the suppression of LDHA and MMPs in liver tumors, consequently leading to inhibition of the progression of NAFLD-driven HCC. Together, our results suggest that BCX feeding improves the hypoxic tumor microenvironment independent of BCO1/BCO2 by increasing acetylated-p53 in the liver tumors, thus contributing to the reduction in progression of NAFLD-driven HCC. However, the underlying mechanisms by

which BCX regulates the hypoxic tumor microenvironment by acetylated-p53 remain undefined and warrant further investigations.

In the current study, BCX feeding significantly reduced protein levels of the anti-apoptotic protein Bcl-xl, but we did not observe a dramatic effect on levels of the anti-apoptotic protein Bax, in the liver tumors of both WT and DKO mice. It has been reported that Bcl-xl, rather than Bax, is an independent prognostic factor in HCC [281]. We presume that the BCX-mediated-Bcl-xl reduction induced apoptosis in the hepatic tumors, thus leading to the suppression of HCC progression in both WT and DKO mice. Additionally, CyclinD1, a regulator of G0/G1 phase, has been reported to be overexpressed in HCC [282], and cyclinD1 inhibition was found to lead to cell cycle arrest, suppression of cell proliferation, and induction of cell apoptosis. We noted that BCX feeding significantly reduced cyclin D1 protein in liver tumors of the WT mice, with a trend in the DKO mice; the absence of such effect may be due to the small sample size for studying tumors. Recent studies have reported that the aberrant glucose metabolism in cancer is required to meet the metabolic demands of cell proliferation and render cancer cells resistant to apoptosis [283]. Further, proliferation and apoptosis in cancer cells is closely related to increased acetylation of p53 [269]. It is therefore reasonable that increased acetylation of p53 and the shifts to glucose metabolism resulting from BCX feeding may contribute to the suppression of the cell cycle and induction of apoptosis which in turn impacts to HCC progression inhibition in both WT and DKO mice. These results were consistent with findings by Gao et al. who reported that BCX induced G0/G1 arrest, inhibited cyclinD1, and increased p53 expression in human gastric cancer cells [56]. Taken together, these results suggest that BCX feeding inhibits cell proliferation and

cell apoptosis, potentially by acetylation of p53 and shifts of glucose metabolism, thereby leading to suppressed HCC progression in both WT and DKO mice.

Recently, several cell culture studies have suggested that BCO1 and BCO2 functions as tumor suppressor genes: BCO1 inhibition in colon cancer cells was associated with an increase in certain characteristics of colon cancer such as cell migration and invasion [49]; BCO1 overexpression inhibited cancer stemness and metastasis in human neuroblastoma cells [51], and BCO2 overexpression in prostate cancer cells suppressed proliferation and colony formation [50]. In the current study, we did not find significant differences in liver tumor outcomes between WT and BCO1/BCO2 DKO mice injected with DEN and fed HRCD alone. Although this finding may not be universally applicable to other different cancer models, we here provide the first *in vivo* evidence using BCO1/BCO2 ablated mice to demonstrate that BCO1 and BCO2 do not play a major role as tumor promoters or tumor suppressors. Consistent with these findings, there were no significant associations between SNPs of BCO1 and the risk of prostate cancer [37], breast cancer [37], and lung cancer [38] in humans.

The dose of BCX (10 mg/kg diet) utilized in our current study is equivalent to consumption of 0.87 mg BCX/day for human, which can be obtained by consuming 1-2 medium-sized sweet red peppers) [54]. This dose was demonstrated to be within a physiological range [271]. As expected, we found that hepatic retinol and retinyl palmitate were higher in the WT mice-fed with BCX, as compared to HRCD littermates indicating bioconversion of BCX into vitamin A. This was supported by significantly lower hepatic BCX detected in WT mice, as compared with that of the DKO mice-fed with the same amount of BCX. It is likely that the combined effects of hepatic BCX and vitamin A might

have contributed to the chemopreventive effects BCX on HCC progression in WT mice. On the other hand, consistent with our previous findings [271], there were no significant differences in vitamin A levels, and a significant amount of BCX (6.6. fold increase) was found in the livers of DKO mice fed an HRCD supplemented with BCX, when compared to levels in the livers of WT mice fed the same diet and a result of the depletion of the carotenoid cleavage enzymes. Our results suggest that the inhibition of BCX on HRCD-promoted HCC progression in DKO mice may be due to the BCX molecule itself having unique biological function, which requires further investigation.

In conclusion, the current findings provide the first scientific evidence suggesting that BCX inhibits the progression of HRCD-promoted HCC by regulating the hypoxic tumor microenvironment and glucose metabolism in the liver tumors of both WT and DKO mice in acetylated p53-dependent manner. Furthermore, we demonstrated that BCO1 and BCO2 do not function as tumor suppressors nor tumor promoters in HCC. Collectively, our findings strongly suggest that the consumption of fruits and vegetables rich in BCX, such as sweet red peppers, butternut squash and tangerine, may be beneficial for preventing the progression of HCC that is associated with the chronic consumption of highly refined carbohydrates.

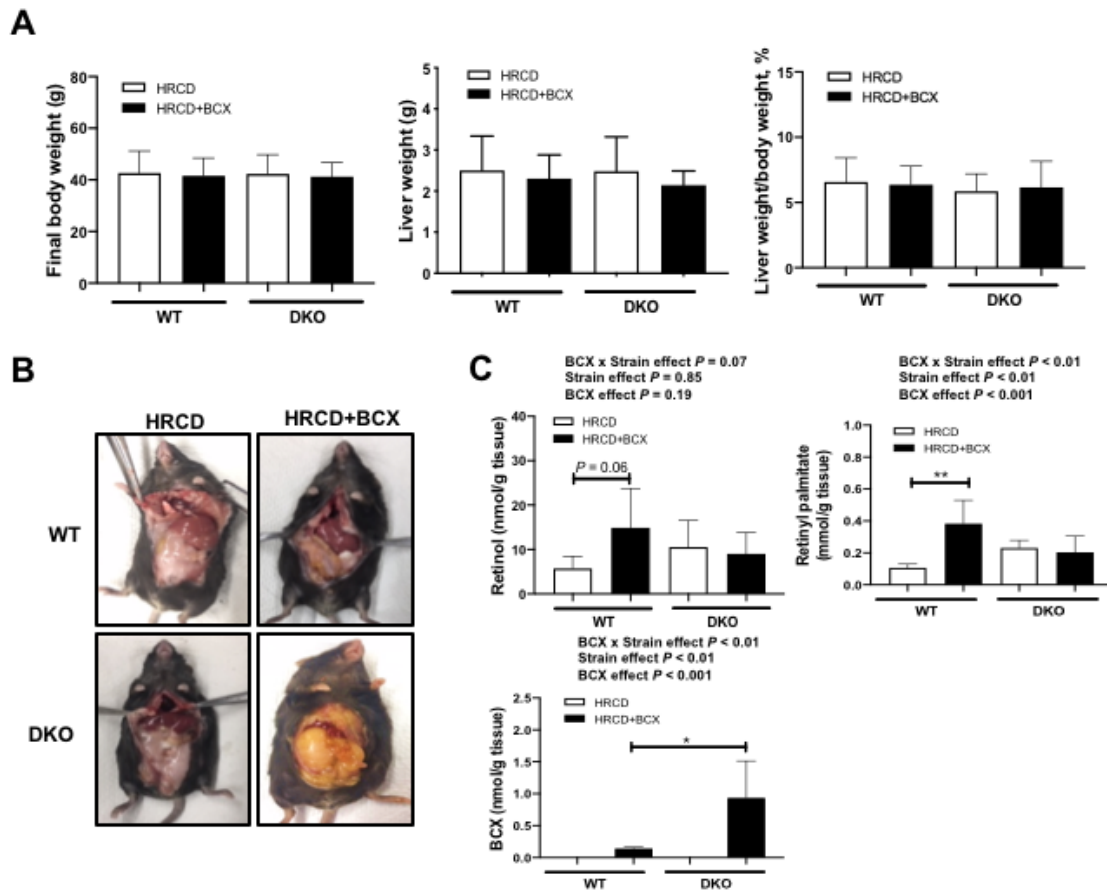


Fig. 1. Analysis of vitamin A and BCX levels in the livers of in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. (A) Final body weight (g), liver weight (g), and liver weight/body weight, %, (B) Shown are close-ups of the internal organs of the mice with significant yellow color of DKO mice fed BCX, (C) retinol, retinyl palmitate, and BCX levels in the livers of WT mice and DKO mice fed HRCD alone or supplemented with BCX. β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; WT, wild-type.

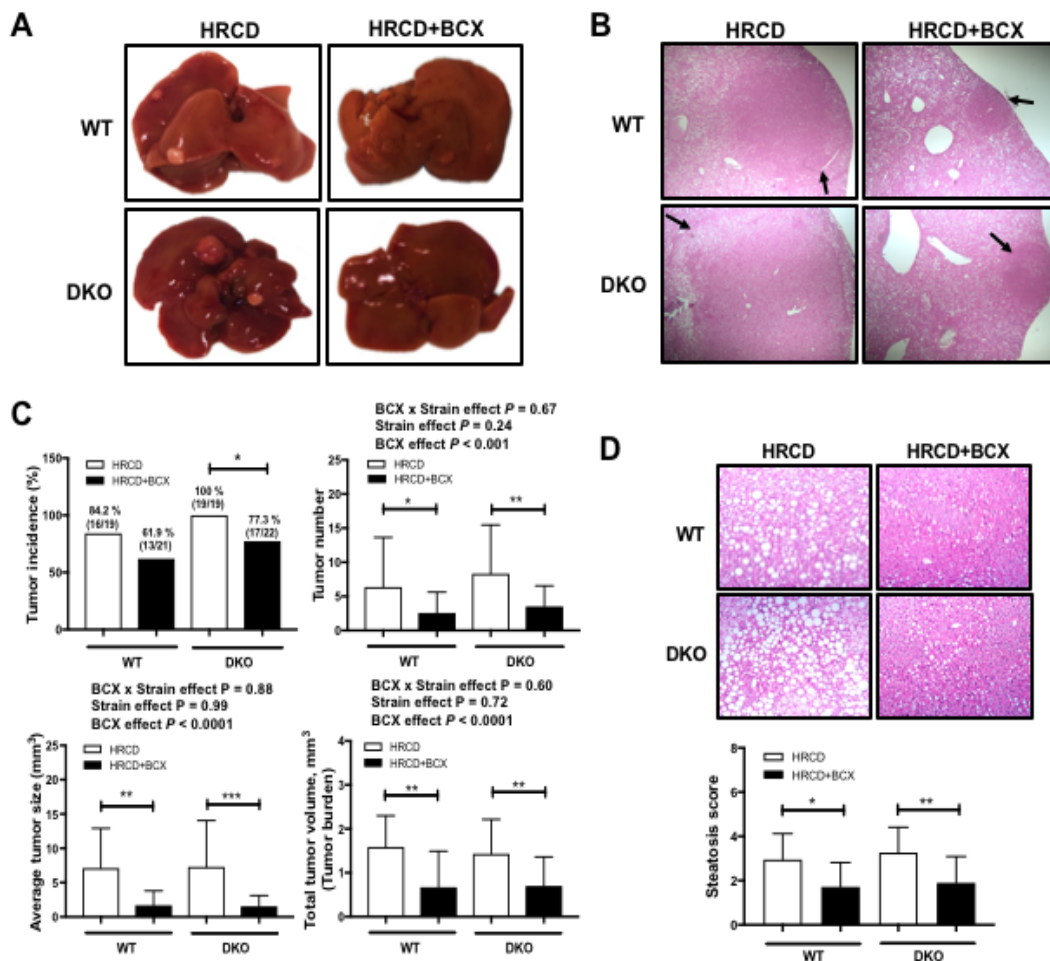


Fig. 2. Histopathological analysis of liver tumors in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. (A) Representative picture of livers from WT mice and DKO mice fed HRCD alone or supplemented with BCX, (B) hematoxylin and eosin-stained liver slides with HCCs (arrows) (C) HCC primary outcomes, (D) hematoxylin and eosin-stained liver slides with hepatic steatosis photographed at 100 $\times$. BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; WT, wild-type.

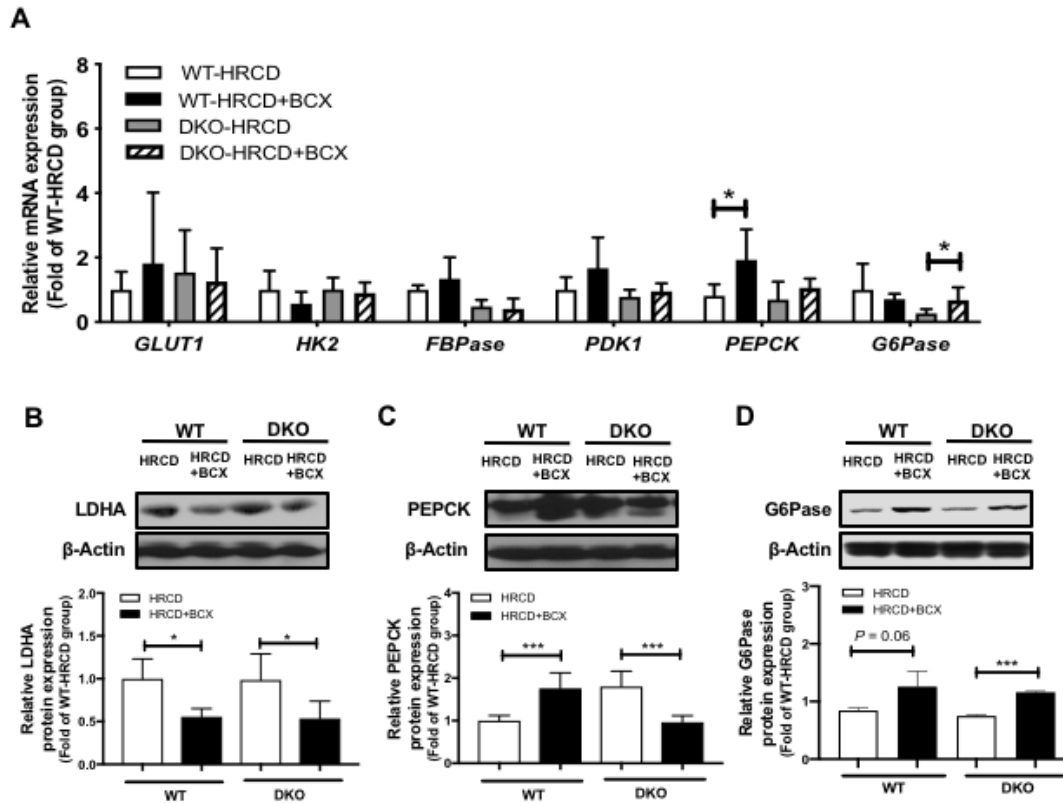


Fig. 3. Effects of BCX on glucose metabolism markers in liver tumors of WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Shown are graphical representations of fold changes in the (A) mRNA expression of glucose metabolism markers, and protein expression of (B) LDHA, (C) PEPCK, and (D) G6Pase quantified via real-time PCR or Western blot in liver tumors of WT mice and DKO mice fed HRCD with or without BCX. β -actin was used as a loading control. Values are means $\pm$ SEM, $n = 3-6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.01$. BCX, β -cryptoxanthin; DKO, double knockout; G6Pase, glucose 6-phosphatase HRCD, high-refined carbohydrate diet; LDHA, lactate dehydrogenase A; PEPCK, phosphoenolpyruvate carboxykinase; SEM, standard error of the mean; WT, wild-type.

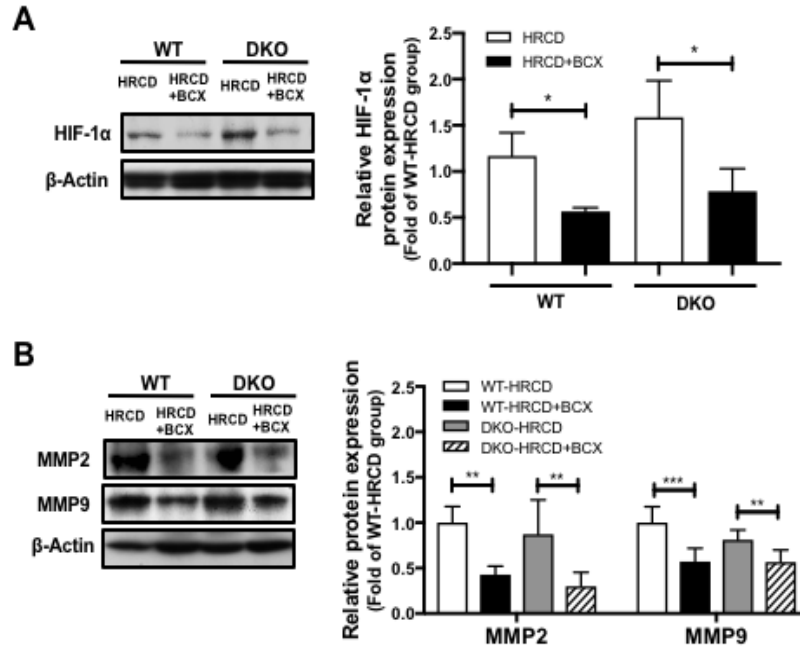


Fig. 4. Effects of BCX on hypoxic tumor microenvironment in liver tumors of WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Shown are graphical representations of fold changes in the protein expression of (A) HIF-1 α , (B) MMP2, and MMP9 quantified via Western blot in liver tumors of WT mice and DKO mice fed HRCD with or without BCX. β -actin was used as a loading control. Values are means $\pm$ SEM, $n = 3-6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.01$. BCX, β -cryptoxanthin; HIF-1 α , hypoxic-inducible factor-1 α ; HRCD, high-refined carbohydrate diet; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; SEM, standard error of the mean; WT, wild-type.

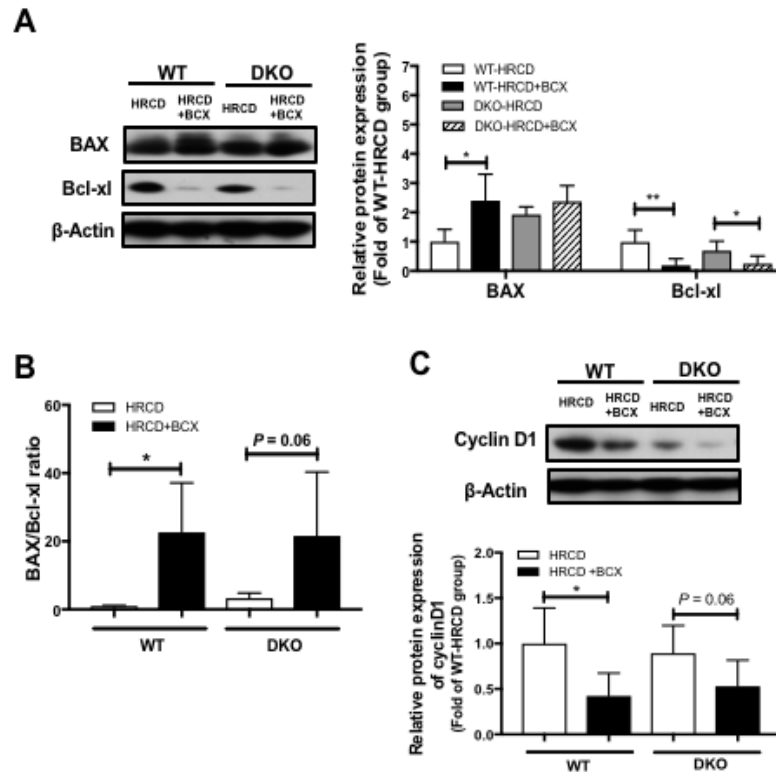


Fig. 5. Effects of BCX on apoptosis markers and cyclinD1 in liver tumors of WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Shown are graphical representations of fold changes in the protein expression of (A) Bax and Bcl-xl, (B) Bax/Bcl-xl ratio, and (C) cyclinD1 quantified via Western blot in liver tumors of WT mice and DKO mice fed HRCD with or without BCX. β -actin was used as a loading control. Values are means $\pm$ SEM, $n = 3-6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.01$. Bax, Bcl-2 associated X; Bcl-xl, B-cell lymphoma-extra-large; BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; SEM, standard error of the mean; WT, wild-type.

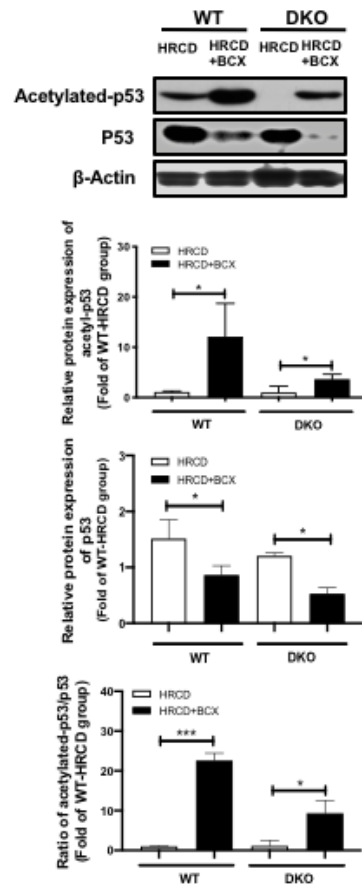


Fig. 6. Effects of BCX on acetylation of p53 in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Shown are graphical representations of fold changes in the protein expression of acetylated-p53, p53, and acetylated-p53/p53 ratio quantified via Western blot in liver tumors of WT mice and DKO mice fed HRCD with or without BCX. β -actin was used as a loading control. Values are means $\pm$ SEM, $n = 3-6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.01$. BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; SEM, standard error of the mean; WT, wild-type.

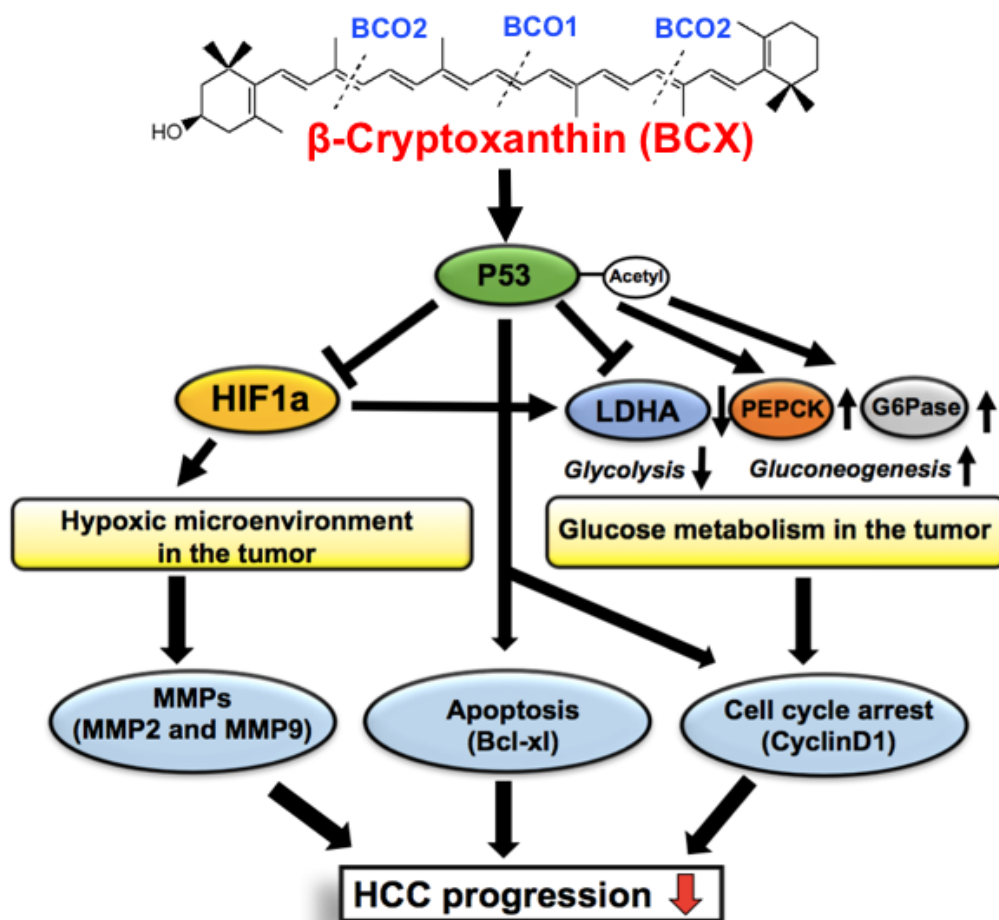


Fig. 7. The proposed underlying mechanisms by which BCX feeding suppresses HRCD-promoted HCC. In WT and DKO mice, BCX feeding increased acetylated p53, subsequently inhibiting HIF-1 α and LDHA in liver tumors. BCX also up-regulates gluconeogenesis by enhancing PEPCCK and G6Pase in liver tumors. Additionally, BCX-mediated inhibition of HIF-1 α suppressed its down-stream targets, MMP2, and MMP9. BCX inhibits cyclinD1 and Bcl-xl potentially through up-acetylation of p53 and glucose metabolism shift, thereby inducing cell cycle arrest and apoptosis in liver tumors of WT and DKO mice. All these factors potentially contribute to suppression of HRCD-promoted HCC. Bcl-xl, B-cell lymphoma-extra-large; BCX, β -cryptoxanthin; G6Pase, glucose 6-phosphatase; HIF-1 α , hypoxic-inducible factor-1 α ; HRCD, high-refined carbohydrate diet; LDHA, lactate dehydrogenase A; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; PEPCCK, phosphoenolpyruvate carboxykinase; WT, wild-type.

Chapter 6: Conclusion, limitations, and future directions

Conclusions

The first part of the current thesis work provided strong experimental evidence that the ablation of both BCO1/BCO2 can lead to the development of NAFLD, thus suggesting that BCO1/BCO2 could play a significant role in maintaining normal hepatic lipid and cholesterol homeostasis, potentially through the regulation of the FXR/miR-34a/SIRT1 pathway. Our results provided new information to the growing body of evidence demonstrating that BCO1 and BCO2 have multiple roles in addition to their carotenoid cleavage activity. Further, our results emphasized the necessity to investigate the relationship between genetic BCO1/BCO2 polymorphisms in humans and the metabolic diseases including NAFLD.

The findings from the second part of this thesis work suggest that BCX exerts protective effects against HRCD-induced NAFLD by regulating multiple molecular pathways in the liver-MAT axis. The molecular mechanisms, however, differ in terms of BCX regulation, which depends on BCO1/BCO2, suggesting that intact BCX has a unique biological function in the prevention of HRCD-induced NAFLD. Together, our results strongly suggest that the consumption of abundant fruits and vegetables enriched in BCX may be suggested to lower the risk of NAFLD associated with the high consumption of refined carbohydrate/sugar diets, regardless of the presence of BCO1/BCO2 polymorphisms in humans.

The findings from the third part of the thesis work provide the first scientific evidence suggesting that BCX inhibits progression of HRCD-promoted HCC by regulating the hypoxic tumor microenvironment and glucose metabolism in the liver tumors of both WT and DKO mice in acetylated p53-dependent manner. Furthermore, we have

demonstrated that BCO1 and BCO2 do not function as tumor suppressors, nor as tumor promoters in HCC. Collectively, our findings strongly suggest that the consumption of fruits and vegetables rich in BCX, such as sweet red peppers, butternut squash and tangerine, may be beneficial for preventing HCC progression associated with the chronic consumption of highly refined carbohydrates.

Limitation of this thesis projects and future directions

In this thesis, we used a BCX concentrate, which was extracted/purified from paprika and dissolved in corn oil. Although we confirmed that this concentrate contained 12.5% BCX and there were no other carotenoids detectable by HPLC, it is still possible that it may contain a trace amount of other biological compounds. In future studies, we will use purified BCX to compare the effects of BCX concentrate and purified BCX compounds on NAFLD and HCC.

Additionally, another limitation of this study is difficulty to determine whether BCX-mediated HCC inhibition comes from BCX-mediated suppression of hepatic lipid accumulation, which requires more comprehensive experiments to confirm the current results and further identify the exact underlying mechanism. A significant number of studies indicate that obesity and increased TG increased free fatty acids, which may contribute to cancer progression. It has been reported that aggressive breast cancer cell lines exhibited higher levels of exogenous free fatty acid uptake marker, CD36, and lipolysis markers [284]. These studies suggest that lipid metabolism of cancer cells is regulated by the availability of fatty acids outside cancer cells [285]. Exogenous lipid uptake by CD36 can promote cancer growth [285]. It is possible that BCX-mediated

inhibition of lipid accumulation in the tumor surrounding region can limit exogenous lipid provision for cancer growth. In the future, it would be interesting to investigate whether BCX supplementation affects the expression levels of fatty acid uptake transporter marker in the tumors and the expression levels of lipolysis markers in tumor surrounding tissues.

Here, we found that BCX up-regulated acetyl-p53 in the liver tumor tissues of WT and DKO mice. However, we do not know the exact underlying mechanisms. We hypothesized that BCX-mediated lipid β -oxidation in the liver tumors may produce higher levels of acetyl-CoA [286], which may lead to the up-regulation of acetyl-p53 [286]. It is known that lipid β -oxidation enhance gluconeogenesis. In line with our hypothesis, we found that BCX feeding up-regulated gluconeogenesis in the liver tumors of WT and DKO mice. It would be interesting to examine whether BCX supplementation regulates lipid β -oxidation and produces total acetyl-CoA levels in the liver tumors.

BCX can be metabolized not only by carotenoid cleavage enzymes but also by microbiota in the gut [287]. We cannot exclude the possibility that the unidentified metabolites in WT and DKO mice in the cancer model can be induced by BCX-mediated gut microbiota change. Recently, we have demonstrated that tomato powder feeding significantly inhibited hepatic inflammation, HCC development, and increased gut microbial diversity in BCO1/BCO2 DKO mice [288]. It is possible that BCX affects gut microbial richness and composition, which can ultimately affect HCC inhibition. Therefore, future studies regarding the effect of BCX on gut microbiome are necessary [289].

It is well recognized that inflammation plays a vital role in the progression of NAFLD to HCC. However, we did not find that HRCD induced inflammatory foci in the liver tissues. This might be due to the discrepancy between mice and humans, which makes it difficult

to apply our results to humans. Thus, future prospective human studies are needed to investigate the effects of BCX using BCX-enriched juice on the metabolic-syndrome-related HCC development as well as for looking into its underlying mechanisms are needed in the future.

Our study showed that BCX supplementation regulates glucose metabolism and hypoxic tumor microenvironment in HCC in both WT and BCO1/BCO2 DKO mice. Moreover, our preliminary data showed that BCX can suppress cancer stem cell marker CD133 in the liver tumors of WT and BCO1/BCO2 DKO mice (Fig. 2b), suggesting that BCX itself plays a unique role in inhibiting cancer stemness in HCC. Similarly, β -carotene, another pro-vitamin A carotenoid, has been shown to have the ability to inhibit neuroblastoma tumorigenesis by modulating cancer stem cell markers including Oct3/4 and delta-like 1 homologue (DLK1) and hypoxic tumor microenvironment marker HIF-1 α in neuroblastoma tumors [144]. Moreover, β -carotene effectively regulated the levels of DLK1 and epithelial mesenchymal transition (EMT), antioxidant enzymes in the normal tissue surrounding tumor tissues, suggesting that β -carotene affects tumor microenvironment unfavorably for tumor growth [145]. In this regard, whether BCX mediated-inhibition of lipid accumulation in the surrounding tissue of hepatic tumor or lipid droplets in the tumor directly affects the development of hepatic tumor growth is unclear and the underlying mechanisms, such as fatty acid delivery to tumors, are currently unknown. Moreover, it remains unknown whether BCX supplementation affects the inflammatory response (NF- κ B pathway) in the normal tissue proximal to the tumor and tumor tissues and how it affects cancer stem cell characteristics (Fig. 1). Natural killer (NK) cells are a major target in immunotherapy owing to their ability to detect and remove

CSCs [290, 291]. Previously, it has been demonstrated that BCX oral administration (1.5 µg/kg for one week) significantly reversed a stress-induced decrease in the cytotoxic activity of NK cells in mice, demonstrating a potential effect of BCX on NK cells [292] (Fig. 1). However, investigating the effect of BCX on NK cells in a cancer murine model is necessary in the future. Unraveling these questions will be helpful when considering BCX as a potential dietary bioactive treatment and immunotherapy aid for cancers. Further detailed investigations elucidating how BCX prevents the development/progression of cancers in animal models are needed to provide insight into the future usage of BCX as a potential bioactive component either by itself or together with cancer treatment medications.

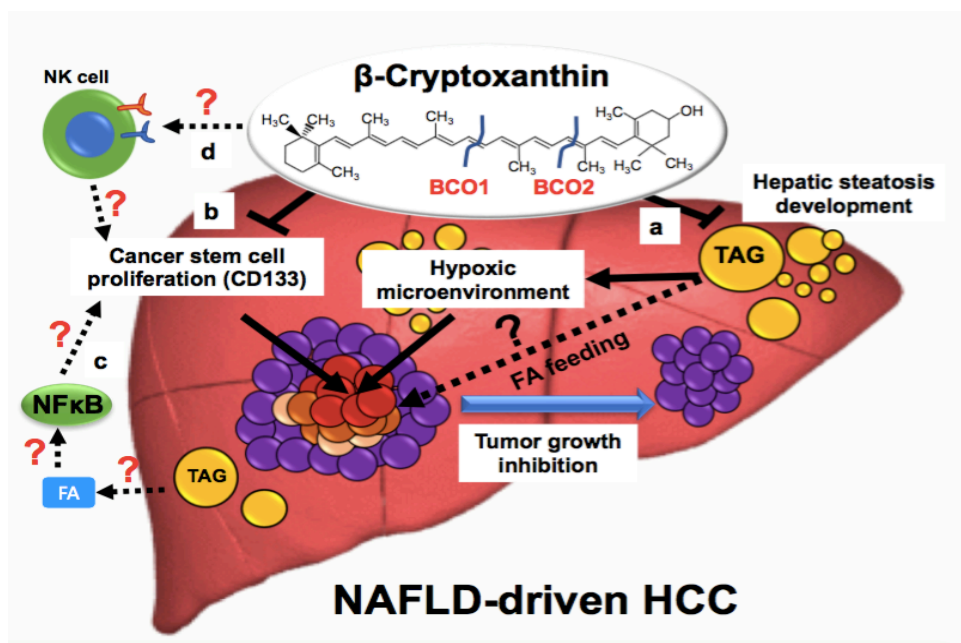


Figure 1. Lipid droplets, cancer stem cells (CSCs), and CSC niche in cancer. (a) BCX supplementation improves hepatic lipid accumulation in non-tumor tissues, and reduces HIF-1α protein expressions in liver tumors in both WT and BCO1/BCO2 DKO

mice; (b) BCX treatment also inhibits cancer stem cell marker, CD133 in the liver tumors in both WT and BCO1/BCO2 DKO mice; (c) BCX supplementation has proven to improve cytotoxic activity of NK cells, which are negative regulators of CSCs, in mice; (d) it remains unclear whether BCX affects the inflammatory response (NF- κ B pathway) in the normal tissue proximal to the tumor, thereby affecting CSCs.

Although we found the beneficial effects of BCX on HRCD-induced NAFLD and HCC, we need to be cautious to use high dose of BCX to treat NAFLD and HCC in humans. It is reported that high-dose of β -carotene supplementation may increase the risk of lung cancer among former smokers or smokers [293]. This study indicates that using high-dose of carotenoids may increase the harmful effects in cancer development. Future studies are warranted to investigate the protective effects of consumption of fruits and vegetables enriched in BCX, such as sweet red peppers and butternut squash against the risk of liver cancer. It would be also interesting to see the consumption of physiological dose of purified BCX supplementation can be used as a chemotherapeutic compound against the progression of HCC in humans.

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Ablation of carotenoid cleavage enzymes (BCO1 and BCO2) induced hepatic steatosis by altering the farnesoid X receptor/miR-34a/sirtuin 1 pathway

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ABSTRACT

β -Carotene-15, 15'-oxygenase (BCO1) and β -carotene-9', 10'-oxygenase (BCO2) are essential enzymes in carotenoid metabolism. While BCO1/BCO2 polymorphisms have been associated with alterations to human and animal carotenoid levels, experimental studies have suggested that BCO1 and BCO2 may have specific physiological functions beyond the cleavage of carotenoids. In the present study, we investigated the effect of ablation of both BCO1/BCO2 in the development of non-alcoholic fatty liver disease (NAFLD) and its underlying molecular mechanism(s). BCO1/BCO2 double knock out (DKO) mice developed hepatic steatosis (8/8) and had significantly higher levels of hepatic and plasma triglyceride and total cholesterol compared to WT (0/8). Hepatic changes in the BCO1/BCO2 DKO mice were associated with significant: 1) increases in lipogenesis markers, and decreases in fatty acid β -oxidation markers; 2) upregulation of cholesterol metabolism markers; 3) alterations to microRNAs related to TG accumulation and cholesterol metabolism; 4) increases in an hepatic oxidative stress marker (HO-1) but decreases in anti-oxidant enzymes; and 5) decreases in farnesoid X receptor (FXR), small heterodimer partner (SHP), and sirtuin 1 (SIRT1). The present study provided novel experimental evidence that BCO1 and BCO2 could play a significant role in maintaining normal hepatic lipid and cholesterol homeostasis, potentially through the regulation of the FXR/miR-34a/SIRT1 pathway.

1. Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD), the most common form of chronic liver disease [1], has dramatically increased in the last 40 years and has been associated with the increased prevalence in obesity [2] of the general population. The spectrum of NAFLD is broad and varies from simple hepatic triglyceride accumulation to non-alcoholic steatohepatitis (NASH) which can progress to terminal hepatic diseases such as cirrhosis or liver cancer [3]. 'Lean NAFLD' (NAFLD in the absence of obesity) has been described in at least 5% of the US population and has been identified in most Western countries [4]. Multiple etiological factors including: high fat and fructose intake and genetic predisposition/risk factors have been shown to contribute to non-obese NAFLD [4]. Numerous single nucleotide polymorphism (SNP) variants associated with lipid metabolism, inflammation, oxidative stress, and insulin resistance have been suggested as potential genetic factors contributing to the development of NAFLD [5]. Thus, it is critical to identify the molecular mechanisms and potential

genetic variants that may result in a susceptibility to NAFLD so as to understand the potential for the prevention and treatment of the development of NAFLD.

In mammalian tissues, β -carotene-15,15'-oxygenase (BCO1), which cleaves carotenoids such as β -carotene and β -cryptoxanthin at the 15,15' double bond, is essential for vitamin A production and homeostasis [6]. β -Carotene-9',10'-oxygenase (BCO2) cleaves carotenoids at the 9,10 double bond to generate the biologically active apo-10'-carotenoids [7]. Previous studies have suggested that SNPs in the human BCO1 gene are common and are associated with reduced catalytic activity in the conversion of β -carotene to vitamin A [8,9]. The rs6564851 SNP of BCO1 is particularly critical for those at risk of vitamin A deficiency [10]; further, this SNP has been associated with high levels of plasma HDL in healthy subjects [11] but there was no association observed between this SNP in BCO1 and risk of breast cancer [12] or type 2 diabetes [13]. The Carotenoids in Age-Related Eye Disease Study (CAREDS) has reported that the rs2250417 SNP in BCO2 was strongly related to age-related eye disease [14]. The rs2115763 SNP of BCO2

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was associated with increased interleukin-18 (IL-18) concentrations [15].

BCO1 and BCO2 knockout (KO) mice present phenotypes, in addition to accumulation of carotenoids in tissues, suggest that these enzymes likely have broader roles resulting in a myriad of physiological changes [16,17]. Both BCO1 and BCO2, with different subcellular localization, are highly expressed in the liver and other peripheral tissues [18,19]. Studies using BCO1 KO mice indicate that BCO1 has a role in the development of hepatic steatosis [20], and in obesity [20], lipid metabolism [20], heart metabolism and function [21], serum insulin, leptin, cholesterol [22], steroid metabolism [23], testosterone synthesis and prostatic cellular proliferation [24]. BCO2 ablation has been shown to result in mitochondrial dysfunction, oxidative stress [16,25], anemia [17], metabolic alteration, liver steatosis [26], and prostate cancer progression [27]. It has been reported that ablating BCO1 has resulted in over-expression of BCO2 [28,29], and carotenoid accumulation influenced whole-body respiration and energy expenditure in the absence of both BCO1 and BCO2 [22,30]. In the present study, we examined if the hepatic phenotype resulting from the lack of both BCO1 and BCO2 contributes to NAFLD development.

The nuclear hormone farnesoid X receptor (FXR), which is mainly expressed in the liver, has been shown to regulate lipogenesis, cholesterol homeostasis, and oxidative stress by controlling downstream gene expression [31–33]. Constitutively active FXR leads to improvement of hyperlipidemia and hyperglycemia [31]. Sirtuin1 (SIRT1), a NAD⁺-dependent histone/protein deacetylase, has been demonstrated to be involved in the regulation of the development of liver steatosis, oxidative stress, and insulin resistance [34,35]. MicroRNAs (miRNAs), known as endogenous small noncoding RNA, are involved in the post-transcriptional regulation of gene expression [36,37] and are aberrantly expressed in many diseases including NAFLD [38]. MicroRNA-34a (miR-34a) is the most abundant hepatic miRNA found in obese mice and NAFLD patients [36,37] and has emerged as an important regulator of SIRT1. Recently, a new regulatory network, the FXR/miR-34a/SIRT1 pathway, has been proposed [39]. The activation of this pathway has been shown to be impaired in obese mice and is associated with a marked increase in hepatic lipid accumulation and liver steatosis [39], suggesting FXR/miR-34a/SIRT1 pathway as a potential novel target for the prevention and treatment of NAFLD. It is however unclear whether BCO1/BCO2 affects this pathway thereby contributing to NAFLD development.

In this present study, we utilized WT and BCO1/BCO2 double KO (DKO) mice fed a standard-chow diet to examine the genetic impact of BCO1 and BCO2 enzymes on the development of NAFLD, oxidative stress, lipid metabolism and the FXR/miR-34a/SIRT1 pathway.

2. Material and methods

2.1. Animals and diets

BCO1^{-/-}/BCO2^{-/-} double knockout mice with C57/BL6; 129Sv mixed genetic background were provided by Dr. J. von Lintig at Case Western Reserve University [30]. Mice were tested with a Mouse 384 SNP panel (Jackson Laboratories, Bar Harbor, ME) to determine C57BL/6J genetic background revealed 83% C57BL/6J genetic background. We then backcrossed the mice onto a C57BL/6J background to generate BCO1^{+/-}/BCO2^{+/-} heterozygous F1 generation mice (estimated to have a 93% C57BL/6J genetic background). The F1 mice were bred to generate both BCO1^{-/-}/BCO2^{-/-} Double Knockout offspring (BCO1/BCO2 DKO) and their corresponding wild type (WT) littermates for the study. Male mice develop hepatic steatosis at dramatically higher rates than female mice, and we previously observed 90% steatosis in male BCO2^{-/-} mice and no steatosis in female mice [40]. Therefore, only male mice were studied in the present study. All experimental protocols and procedures were approved by the Tufts University Institutional Animal Care and Use.

All mice were fed ad libitum chow diet which contained 15000 IU vitamin A/kg diet (Teklad 2916.15, Envigo, Madison, WI) for 26 weeks, provided free access to water, and were maintained under environmental conditions of 22 ± 2 °C, 40 ± 5% humidity and 12/12 light/dark cycle. During the experimental period, food intake was monitored and all animals were weighed twice weekly. Euthanasia was performed at 30 weeks of age consistent with findings by Hessel et al. [20] who demonstrated that BCO1^{-/-} KO mice fed standard chow diet had no hepatic steatosis at 4 weeks old but was observed with an increased prevalence with advancing age (12 and 24 weeks of age). Further, hepatic triglyceride levels were significantly higher in the livers of 24 weeks-old BCO1^{-/-} KO mice. Liver tissues were harvested post-mortem and weighed. The left lobe of mouse liver was fixed in 10% buffered formalin solution (Thermo Fisher Scientific, USA). The remaining sections of liver were divided into smaller portions, snap-frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

2.2. Liver histopathology evaluation

Five-micrometer sections of formalin-fixed and paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E, Sigma Aldrich, St Louis, MO, USA) for histopathological analysis, as previously described [41,42]. Sections were examined under light microscopy by two independent investigators who were blinded to the treatment groups. A Zeiss microscope with a PixeLINK USB 2.0 (PL-B623CU) digital Camera and PixeLINK µScope Microscopy Software was used for image capture and quantification for all histopathological analyses. Hepatic steatosis was graded according to steatosis magnitude (both macro- and micro-vesicular fat accumulation). Briefly, the degree of steatosis was graded based on the percentage of the liver section that was occupied by fat vacuoles at 100 × magnification in 20 fields (grade 0 ≤ 5%; grade 1 = 5–25%; grade 2 = 26–50%; grade 3 = 51–75%; grade 4 = 76–100%). Hepatic steatosis incidence was determined by the percentage of mice with a hepatic steatosis score greater than 0.

2.3. Reverse transcription-PCR and quantitative RT-PCR

Total RNA was prepared from 50 to 100 mg samples of frozen liver tissue using a TriPure Isolation Reagent kit (Roche, Indianapolis, IN, USA). Briefly, deep-frozen liver tissues were minced and homogenized with Tripure Isolation Reagent (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. cDNA was synthesized by reverse transcription using M-MLV (Invitrogen, Carlsbad, CA, USA) and was used for quantitative real-time PCR analysis. Quantitative real-time PCR was performed using specific primers and FastStart Universal SYBR Green Master (Roche, Indianapolis, IN, USA). The relative expression of all mRNAs were calculated using the comparative threshold cycle (Ct) method [43]. Tissues from genetic background-matched WT littermate mice were used to calibrate samples. For the normalization, the amplification of β-actin was used for loading control. The primers used were listed in [Supplementary Table 1](#).

2.4. MicroRNA quantification by real-time qRT-PCR analysis

Total RNA (100 ng) was prepared from liver tissues to generate cDNA by reverse transcription with Moloney murine leukemia virus RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The relative expressions of all microRNAs were quantified using the comparative threshold cycle (Ct) method as identified above. Tissues from genetic background-matched WT littermate mice were used to calibrate samples. For the normalization, the amplification of U6 for every sample was used.

2.5. Western blot

Western blotting was performed as previously described [44].

Briefly, the protein was extracted from 50 to 100 mg samples of liver tissue by lysing with RIPA lysis buffer. Homogenates were centrifuged at 55,000 rpm for 60 min at 4 °C using an ultracentrifuge and supernatants collected and stored at – 80 °C. Equal aliquots of sample (50 µg) were used and mixed with 2 × reducing Laemmli sample buffer. Denatured protein was applied to 10% SDS-polyacrylamide gels, transferred electrophoretically to Immobilon-P membranes (Millipore, Billerica, MA, USA) and subsequently blocked with 5% non-fat dried milk in Tris-buffered saline containing Tween-20 (TBST) buffer. Membranes were probed with primary antibodies against FXR (GeneTex, Irvine, CA, USA), HO-1 (Santa Cruz, TX, USA), SCD-1, ACC (Cell Signaling, Danvers, MA, USA), and β-actin (Sigma-Aldrich) at 4 °C overnight. After being washed three times with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA) in 5% non-fat milk. All membrane-bound primary antibodies were examined by a SuperSignal West Chemi-luminescent Substrate Kit (Pierce, Rockford, IL, USA) and the specific relative band intensities were quantified by scanning with a model GS-710 Calibrated Imaging Densitometer (Bio-Rad). For normalization, β-actin was used as loading control.

2.6. Liver and plasma triglyceride (TG) and total cholesterol (TC) analysis

Hepatic and plasma TG and TC levels were measured using a commercial total cholesterol assay kit (Cell Biolabs Inc, San Diego, CA, USA) and TG colorimetric assay kit (Cayman, Ann Arbor, Michigan, USA). All procedures followed manufacturer's protocols. TG and TC concentrations were measured at 540 nm.

2.7. High-performance liquid chromatography (HPLC) analysis

Liver samples for HPLC analysis were prepared as previously described [45]. Analysis of retinol, retinyl palmitate, and lutein levels in liver tissue was performed using gradient reversed phase HPLC consisting of a Waters 2996 photodiode array detector (Waters), a Waters 2695 separation module and a reverse-phase C18 column (4.6 × 250 mm, 5 µm) with a flow rate of 1.00 mL/min, and was quantified by determining peak areas in chromatograms calibrated against known amounts of standard.

2.8. Statistical analysis

All results were represented as the mean ± standard error of the mean (SEM). The two-tailed, unpaired Student's *t*-test was used to compare TG, TC, gene and protein expression between two groups. The Mann-Whitney test was used for the liver steatosis score distribution comparison. The analysis was conducted using GraphPad-Prism 7.0 software (GraphPad Software, Inc.) and *P* < 0.05 was considered significantly different.

3. Results

3.1. Ablation of BCO1 and BCO2 increases lipid accumulation in the liver

There were no significant differences in final body weight, liver weight, and mesenteric fat weight between WT and BCO1/BCO2 DKO mice (Table 1). Histological analysis revealed that 100% (8/8) of mice in the BCO1/BCO2 DKO group developed hepatic steatosis (score 1), whereas hepatic steatosis was not observed in WT animals (0/8) (*P* < 0.001) (Table 1). Liver histopathological examination demonstrated that large and small lipid vacuoles were markedly increased in the BCO1/BCO2 DKO mice (*P* < 0.001) compared to WT mice (Fig. 1). To confirm these histological findings, TG and TC levels in both liver and plasma were examined: BCO1/BCO2 DKO mice had approximately 50% higher hepatic TG (*P* < 0.05) and 75% higher hepatic TC (*P* < 0.01) levels compared to the WT mice (Table 1). Although there

Table 1
Primary outcomes.

Experimental group	WT	BCO1/BCO2 DKO	<i>P</i> -value
Number of mice	8	8	
Body weight (g)	33.15 ± 2.63	37.27 ± 6.80	0.065
Liver weight (g)	1.47 ± 0.14	1.87 ± 0.75	0.086
Liver weight/Body weight, %	4.44 ± 0.26	4.49 ± 0.99	0.14
Mesenteric fat weight (g)	0.46 ± 0.081	0.56 ± 0.24	0.199
Mesenteric fat weight/Body weight, %	1.37 ± 0.16	1.4 ± 0.38	0.53
Liver steatosis incidence, % of mice	0% (0/8)	100% (8/8)***	< 0.001
Hepatic steatosis score, median (range)	0 (0–0)	1 (1–1)***	< 0.001
Hepatic lipids (mg/g tissue)			
TG level (mg/g)	2.83 ± 0.78	4.26 ± 1.60*	< 0.05
TC level (mg/g)	1.60 ± 0.48	2.81 ± 0.28**	< 0.01
Plasma lipids (mg/dL)			
TG level (mg/dL)	113.1 ± 22.08	95.1 ± 16.01	0.082
TC level (mg/dL)	33.61 ± 8.01	51.98 ± 4.08**	< 0.01
Hepatic retinol (nmol/g)	15 ± 4.9	40 ± 6	0.34
Hepatic retinyl palmitate (nmol/g)	1120 ± 38	1020 ± 75	0.22
Hepatic lutein (nmol/g)	N/D	1.22 ± 0.47	

AbbreviationsTG, triglycerides; TC, total cholesterol; N/D, not detected. Values are presented as means ± SEM of 8 mice. Statistical significance was determined by the unpaired *t*-test. To compared liver steatosis score, Mann-Whitney test was used. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

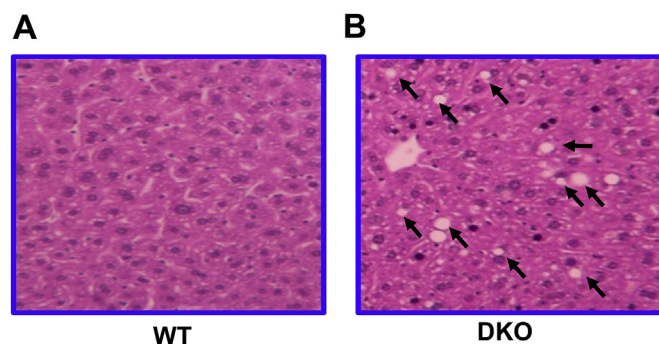


Fig. 1. Hepatic steatosis in BCO1/BCO2 DKO mice. (A) WT, (B) BCO1/BCO2 DKO. Hematoxylin and eosin stained slides were photographed at a magnification of 100x.

were no changes in plasma TG, the plasma TC levels were significantly (54%) higher in BCO1/BCO2 DKO mice (*P* < 0.01) (Table 1). Both hepatic TG and TC levels were significantly and positively correlated with liver weight/b. w ($R^2 = 0.316$, *P* = 0.012). These results indicate that ablation of both BCO1 and BCO2 genes lead to lipid (TG and TC) accumulation in the liver.

3.2. Retinol, retinyl palmitate, and lutein levels

There were no significant differences in retinol or retinyl palmitate levels between the two experimental groups (Table 1). Lutein was not detected in the livers of WT mice but was observed in small amounts in the livers of BCO1/BCO2 DKO mice (1.22 ± 0.47 nmol/g tissue) (Table 1). We did not observe any accumulation of other carotenoids or their metabolites in either WT or BCO1/BCO2 DKO mice.

3.3. Ablation of BCO1 and BCO2 increases de novo hepatic lipogenesis and inhibits lipid β-oxidation

In order to examine the gene expression of mRNAs and proteins related to lipogenesis and fatty acid β-oxidation in WT and BCO1/BCO2 DKO mice, Western blot and qPCR were performed. We found that the

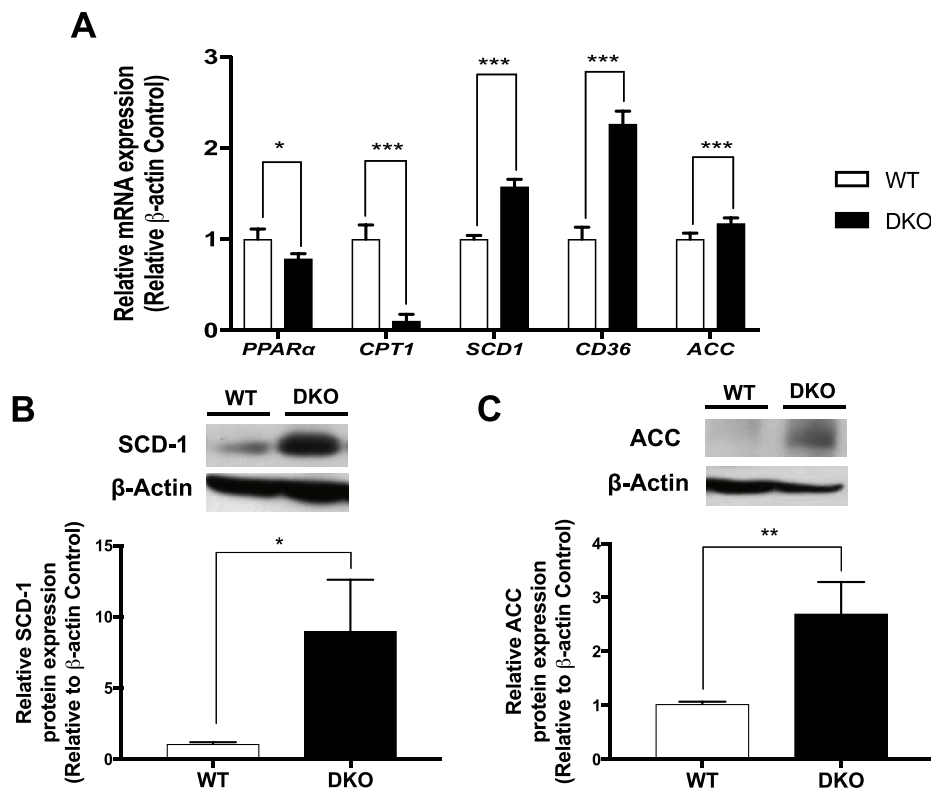


Fig. 2. The effects of BCO1/BCO2 DKO on lipogenesis and fatty acid β -oxidation biomarkers. (A) Relative mRNA levels of fatty acid synthesis markers (*SCD1*, *ACC*, *CD36*) and fatty acid β -oxidation markers (*PPARα*, *CPT1*) were detected by qPCR in the livers of WT and BCO1/BCO2 DKO mice. β -actin was used as a loading control. (B) Relative SCD1 protein levels, (C) Relative ACC protein levels were analyzed by western blotting of liver lysates of WT and BCO1/BCO2 DKO mice. Representative results are shown in upper panels and band intensities were quantified by densitometry. β -actin was used as a loading control. Statistical significance was determined by the unpaired *t*-test. Values are mean $\pm$ SEM (*n* = 3–6 per group). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

stearoyl-CoA desaturase 1 (*SCD1*) and acetyl-CoA carboxylase (*ACC*) gene expression in the liver were significantly increased by 57% and 17%, respectively, in BCO1/BCO2 DKO mice compared to the WT mice (*P* < 0.05) (Fig. 2A). We confirmed that the relative protein levels of *SCD1* by western blot were approximately 9-fold higher in BCO1/BCO2 DKO mice than those in WT mice (*P* < 0.05) (Fig. 2B). Further, *ACC* protein levels were approximately 2-fold higher in BCO1/BCO2 DKO mice compared to the WT mice (*P* < 0.01) (Fig. 2C). Hepatic gene markers for fatty acid oxidation, such as carnitine palmitoyltransferase 1 (*CPT1*) and peroxisome proliferator-activated receptor alpha (*PPARα*), were significantly inhibited by 90%, and 22% respectively (*P* < 0.05) (Fig. 2A). Hepatic mRNA levels of *CD36* (involved in fatty acid uptake) were up-regulated 2.5-fold in BCO1/BCO2 DKO mice (*P* < 0.001) (Fig. 2A). These data indicate that the hepatic lipid accumulation in the BCO1/BCO2 DKO mice is associated with elevated *de novo* hepatic lipogenesis and decreased fatty acid β -oxidation.

3.4. Ablation of BCO1 and BCO2 alters cholesterol metabolism

To explain the high TC levels in BCO1/BCO2 DKO mice, we investigated markers related to cholesterol metabolism. qPCR results revealed that mRNA levels of cholesterol 7 α -hydroxylase (*CYP7A1*) and sterol 12 α -hydroxylase (*CYP8B1*), involved in bile acid synthesis from cholesterol, were significantly increased in BCO1/BCO2 DKO mice (*P* < 0.05) (Fig. 3). Furthermore, gene expression of hepatic 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (*HMG-CoAR*), a rate-controlling enzyme of cholesterol synthesis, in BCO1/BCO2 DKO mice increased by 40% compared to the WT mice (*P* < 0.001) (Fig. 3). These data suggest that the BCO1/BCO2 DKO induced cholesterol synthesis by elevating *HMG-CoAR* and cholesterol utilization by *CYP7A1* and *CYP8B* (which requires cholesterol as the precursor molecule for bile acid synthesis).

3.5. Ablation of BCO1 and BCO2 induces oxidative stress in the liver

Because accumulated lipid droplets in the liver can induce

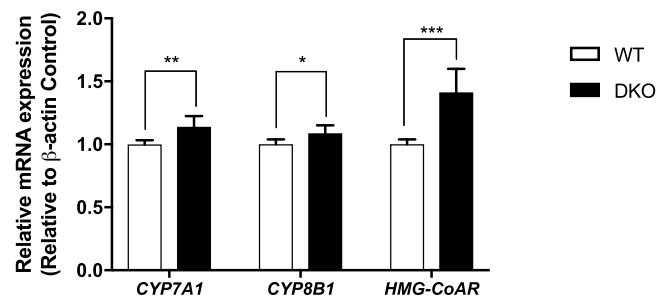


Fig. 3. The effects of BCO1/BCO2 DKO on cholesterol synthesis and bile acid synthesis biomarkers. Relative mRNA levels of cholesterol metabolism markers (*CYP7A1*, *CYP8B1*, *HMG-CoAR*) were determined by qPCR in liver samples of WT and BCO1/BCO2 DKO mice. β -actin was used as a loading control. Statistical significance was determined by the unpaired *t*-test. Values are mean $\pm$ SEM (*n* = 6 per group). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

mitochondrial oxidative stress [46], we examined the anti-oxidant enzymes in the liver of the BCO1/BCO2 DKO. Findings from the Western blots and qPCRs demonstrated a 3-fold up-regulation of protein expression of hepatic heme-oxygenase 1 (*HO-1*) (*P* < 0.01) (Fig. 4A) and a 2-fold induction of *HO-1* mRNA expression (*P* < 0.001) (Fig. 4B) in BCO1/BCO2 DKO mice compared to WT mice. A small but significant reduction in mRNA levels of glutathione peroxidase 1 (*GPX*), superoxide dismutase 1 (*SOD1*), *SOD2*, and catalase (16%, 6%, 13%, and 18%, respectively) was observed in the BCO1/BCO2 DKO mice (*P* < 0.05) (Fig. 4B). These results demonstrated that the BCO1/BCO2 DKO in these mice resulted in an increased oxidative stress and decreased antioxidant activity.

3.6. BCO1/BCO2 DKO causes impairment of the FXR/miR-34a/SIRT1 pathway in the liver

Activation of FXR induces expression of a small heterodimer partner (SHP) that results in suppression of miR-34a, thereby increasing SIRT1

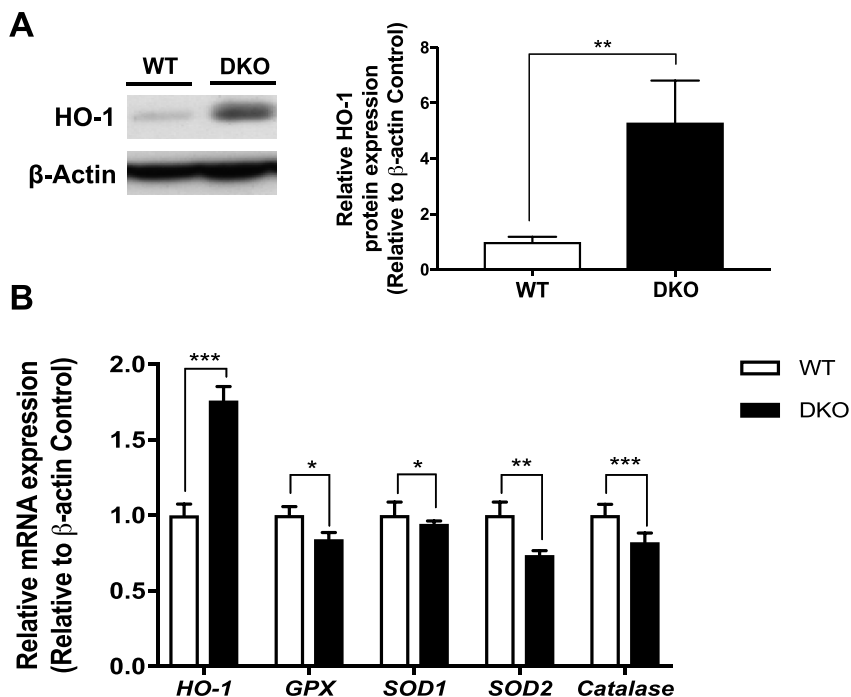


Fig. 4. The effects of BCO1/BCO2 DKO on oxidative stress biomarkers. (A) Relative protein levels of HO-1 were analyzed by western blots of liver lysates of WT and BCO1/BCO2 DKO. (B) Relative mRNA levels of anti-oxidant enzymes (HO-1, GPX, SOD1, SOD2, catalase) were detected by qPCR in liver samples of WT and BCO1/BCO2 DKO mice. Representative results are shown in the left panel. Band intensities were quantified by densitometry. β -actin was used for normalization in qPCR and western blot testing. Statistical significance was determined by the unpaired *t*-test. Values are mean $\pm$ SEM (*n* = 6 per group). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

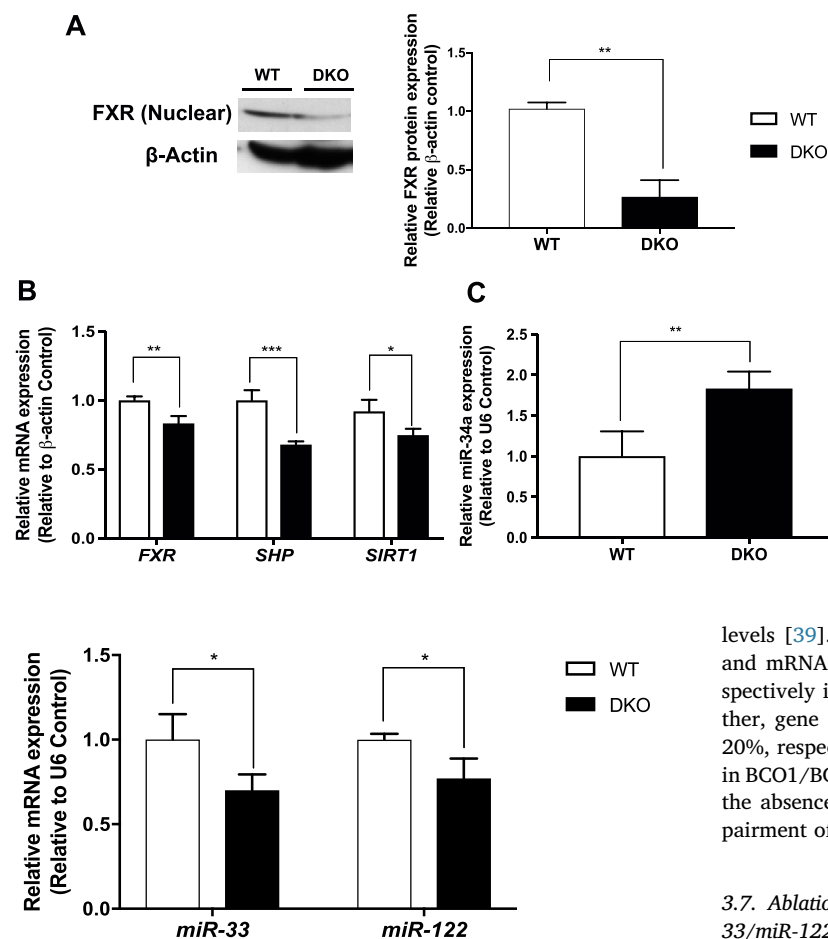


Fig. 5. The effects of BCO1/BCO2 DKO on FXR/miR-34/SIRT1 pathway. (A) Relative protein levels of FXR is analyzed by western blots of nuclear extract of livers of WT and BCO1/BCO2 DKO mice. (B) Relative mRNA levels of FXR, SHP, and SIRT1 were quantified by qPCR. β -actin was used for normalization in qPCR and western blot testing. (C) Relative expression levels of miR-34a is analyzed by qPCR. U6 snRNA was used for normalization. Representative results are shown in the left panel. Band intensities were quantified by densitometry. Statistical significance was determined by the unpaired *t*-test. Values are mean $\pm$ SEM (*n* = 3–6 per group). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Fig. 6. The effects of BCO1/BCO2 DKO on miR-33/miR-122 axis. miR-33 and miR-122 were determined by qPCR of liver samples from WT and BCO1/BCO2 DKO mice. Statistical significance was determined by the unpaired *t*-test. Values are mean $\pm$ SEM (*n* = 4 per group). **P* < 0.05.

levels [39]. The Western blots and qPCR results showed that protein and mRNA FXR levels were significantly reduced 20% and 17%, respectively in BCO1/BCO2 DKO mice (*P* < 0.01) (Fig. 5A and B). Further, gene expression of SHP and SIRT1 was decreased by 32%, and 20%, respectively (*P* < 0.05) (Fig. 5B). miRNA-34a was 2-fold higher in BCO1/BCO2 DKO mice (*P* < 0.01) (Fig. 5C). These data suggest that the absence of BCO1/BCO2 induced NAFLD in the DKO mice by impairment of the FXR/miR-34a/SIRT1 signaling pathway.

3.7. Ablation of BCO1 and BCO2 leads to the aberrant levels of the miR-33/miR-122 axis

MiR-122 and miR-33 are involved in *de novo* lipid synthesis, cholesterol and bile acid homeostasis, and oxidative stress [47–49]. It has been reported that miR-122 and miR-33 are downstream-targets of FXR since they have specific binding sites for FXR [50,51]. We quantified the levels of miR-33 and miR-122 in the liver by qPCR, and observed

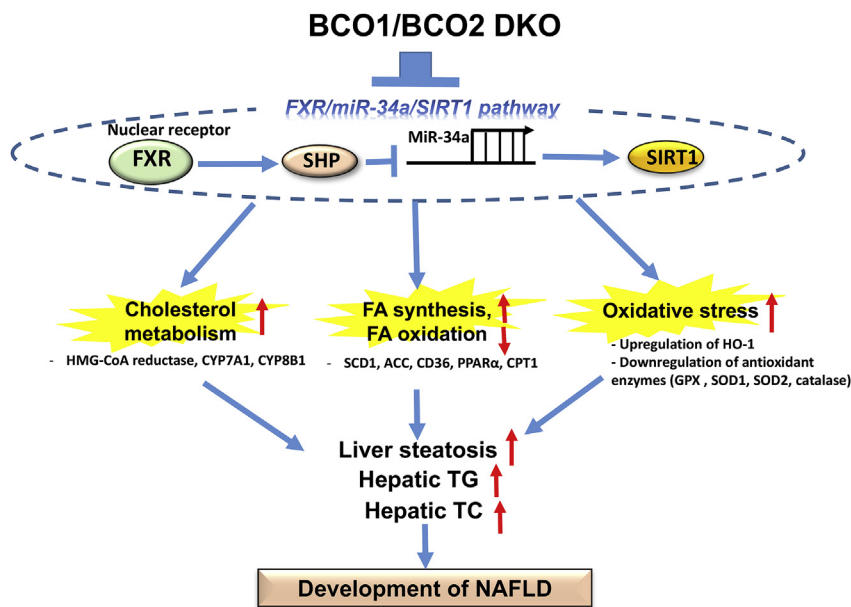


Fig. 7. The proposed mechanism of BCO1/BCO2 ablation induces NAFLD. BCO1/BCO2 DKO inactivates the FXR/miR-34a/SIRT1 pathway, leading to aberrant expression levels of miR-33/miR-122 axis; this directly and indirectly regulates various biomarkers involved in fatty acid synthesis, fatty acid β -oxidation, cholesterol metabolism and oxidative stress. These results demonstrate: 1) elevated hepatic fatty acid synthesis and reduced β -oxidation; 2) increased cholesterol synthesis and elevated cholesterol utilization; 3) increased oxidative stress and reduced anti-oxidant capacity (that may lead to increased hepatic steatosis with elevated hepatic TG and hepatic TC levels) resulting in the development of NAFLD.

that the BCO1/BCO2 DKO mice had a 23% decrease in hepatic miR-122a ($P < 0.05$) (Fig. 6). In addition, hepatic levels of miR-33 in the BCO1/BCO2 DKO mice were 30% lower than those in the WT mice ($P < 0.05$) (Fig. 6). This decrease of miR-33 and miR-122 levels (as the downstream-targets of FXR) in BCO1/BCO2 DKO mice could result from the impairment of the FXR signaling pathway.

4. Discussion

In this current study, we demonstrated that the absence of BCO1 and BCO2 in mice fed a normal chow diet altered normal lipid and cholesterol metabolism and induced oxidative stress. This observation was associated with the alteration of FXR/miR-34/SIRT1 pathway and miR-33/miR-122 axis in the liver and contributing to NAFLD development (Fig. 7). Our findings support the notion that BCO1 and BCO2 enzymes have separate biological functions from carotenoid metabolism and suggest the possibility that genetic BCO1/BCO2 polymorphisms in humans could be an independent risk factor for the susceptibility to NAFLD.

In the current study, we demonstrated that all BCO1/BCO2 DKO mice developed hepatic steatosis when fed a standard chow diet for 26 weeks. In addition to histological changes, there was an increase in hepatic TG, hepatic TC, and plasma TC levels in the mice which may be a result of the combination of both enzyme deficiencies since BCO1 and BCO2 regulate lipid metabolism differently via different regulatory networks [16,20,22,26]. Hessel et al. have demonstrated the development of marked liver steatosis with high hepatic TG in the BCO1 KO mice, suggesting that liver steatosis was directly associated with BCO1 deficiency [20]. Ford et al. have reported that BCO2 KO mice showed higher levels of hepatic cholesterol and total hepatic lipid as compared to WT mice, while no differences were observed in the BCO1 KO mice [22]. Further, histopathological data indicated that hepatic steatosis was observed in 25% of the BCO1 KO mice, but not in the livers of WT or BCO2 KO mice [22]. Elevated oxidative stress markers and dysregulated mitochondrial enzyme activity have been reported in BCO2 KO mice [16]. In the current study, all BCO1/BCO2 DKO mice exhibited both the pathological characteristics previously described for BCO1 and BCO2 KO mice with more severe histopathological features (e.g. 100% of BCO1/BCO2 DKO mice developed liver steatosis) suggesting that deletion of both BCO1 and BCO2 enzymes can inevitably lead to NAFLD.

Impairment of the FXR/miR-34/SIRT1 pathway potentially

mediates the development of liver steatosis by increasing lipogenesis and reducing fatty acid β -oxidation. FXR has been shown to inhibit expression of FAS, SCD-1 and ACC, and prevent overproduction of TG, suggesting a significant effect on lipogenesis [52]. Further, FXR has the ability to enhance TG clearance by inducing PPAR α and its target gene, CTP-1 [53]. We found elevated levels of hepatic lipogenesis markers (including SCD-1 and ACC) in the BCO1/BCO2 DKO mice but levels of fatty acid β -oxidation markers (including PPAR α and CPT1) were reduced. This is consistent with the reports of others that demonstrated decreased levels of markers related to fatty acid β -oxidation including PPAR α in BCO2 KO mice [16]. CD36 is a fatty acid translocase intimately related to hepatic steatosis and hepatic cholesterol uptake is highly expressed in NAFLD patients [54] and Hessel et al. reported that CD36 expression was increased in BCO1 KO mice [20]. In the current study, we observed an increase in hepatic CD36 expression with decreased FXR levels in the BCO1/BCO2 DKO mice. Since FXR has been demonstrated to inhibit liver steatosis by effectively reducing CD36 expression [55], our results suggest that increased CD36 expression may result from decreased hepatic FXR levels in the DKO mice. Further, CD36 overexpression in peripheral tissues reduces plasma fatty acid (FA) and TG levels [56] and we observed that increased CD36 mRNA expression was associated with higher hepatic TG levels ($P < 0.05$) and lower plasma TG levels ($P = 0.08$) in the BCO1/BCO2 DKO mice. We have previously reported that ablation of SIRT1 activity in mice resulted in hepatic steatosis by over-expression of CD36 in the liver [44], suggesting that increased CD36 levels may also be due to suppressed SIRT1 levels in BCO1/BCO2 DKO mice. Further, we previously demonstrated that hepatic steatosis induced by a highly refined carbohydrate diet was mediated by the dysregulation of the miR-34a/SIRT1 axis in mice [57]. We found that the down-regulation of FXR was accompanied with lower expressions of SHP and SIRT1 but higher expression of miR-34a in the BCO1/BCO2 DKO mice, suggesting that both BCO1 and BCO2 play significant roles in regulating fatty acid uptake, *de novo* fatty acid synthesis, and mitochondrial fatty acid β -oxidation via the FXR/miR-34/SIRT1 pathway (Fig. 7). The mechanism(s) underlying the suppression of FXR by BCO1/BCO2 DKO remains unclear, and further studies (such as bile acid analysis) are needed.

In the current study, both hepatic and plasma TC accumulation were increased in the BCO1/BCO2 DKO mice. The rapid increases in cholesterol levels as NAFLD progressed to NASH suggests that TC contributes to NAFLD pathogenesis [58]. Cholesterol homeostasis is maintained by the balance of cholesterol synthesis with cholesterol

efflux (by bile acid synthesis). Our findings indicate that despite a minor elevation in cholesterol utilization by CYP7A1 and CYP8B1, dramatic increases in cholesterol synthesis by HMG-CoA reductase led to the accumulation of TC in both liver and plasma in the DKO mice. These effects are also potentially mediated by the FXR/miR-34/SIRT1 pathway: FXR suppresses CYP7A1 and CYP8B1 (by induction of SHP) to control cholesterol and bile acid levels [59]. Therefore, reduced expression of FXR and SHP may account for increased levels of CYP7A1 and CYP8B1 that led to increases in both hepatic and plasma TC levels in the DKO mice. Further, we observed that HMG-CoA reductase levels were significantly higher in the livers of the DKO mice. A possible mechanism for the elevation of HMG-CoA reductase in the BCO1/BCO2 DKO mice may be the elevation of hepatic miR-34a levels (Fig. 2C). miR-34, a microRNA that is highly expressed in NASH [37], inhibits SIRT1, which can subsequently lead to inactivation of 5'-prime-AMP-activated protein kinase (AMPK), a known inhibitor of HMG-CoA reductase [60,61]. Taken together, our results provide compelling evidence that the cooperative role of BCO1 and BCO2 enzymes are important in the maintenance of cholesterol homeostasis in liver and plasma by regulating expressions of HMG-CoA reductase, CYP7A1, and CYP8B1 by the FXR/miR-34/SIRT1 pathway.

To further examine the down-stream effectors of FXR, we demonstrated that the levels of miR-33 and miR-122 were significantly reduced in the livers of BCO1/BCO2 DKO mice. Others have reported the FXR-enhanced transcription of miR-33 and miR-122 by the direct binding to the specific region of miR-33 and miR-122 [50,51], implying that impaired FXR levels were directly linked to the suppressed miR-33/miR-122 axis. Although miR-33 has been reported to repress major genes in cholesterol export and high-density lipoprotein (HDL) metabolism in short-term studies, a recent study has commented that long-term inhibition of miR-33 led to high TG and liver steatosis in obese mice fed a high fat diet [62]. Similarly, Horie et al. reported that miR-33 KO mice exhibited fatty liver, high TG levels, and obesity [63]. In the current study, we confirmed the finding that miR-122 was suppressed in BCO1/BCO2 DKO mice. Consistent with our results, Hsu et al. demonstrated that miR-122 KO mice exhibited liver micro-steatosis with 2-fold higher TG levels compared to the control [64]. Others have reported that miR-122 inhibition reduced serum cholesterol and lipogenesis [47,65]. It is important to note that transient suppression of miR-122 using oligonucleotides are well tolerated, while prolonged miR-122 deletion leads to hepatic TG accumulation [64]. Inactivation of FXR may lead to prolonged suppression of miR-122 with the development of NAFLD. Altogether, FXR directly or indirectly modulates genes involved in lipid synthesis, lipid β -oxidation, cholesterol metabolism, and oxidative stress by the miR-33/miR-122 axis and induces the development of NAFLD in the BCO1/BCO2 DKO mice.

Oxidative stress exacerbates the pathogenesis of NAFLD and is considered to be the most critical mechanism contributing to hepatic injury in NAFLD [66]. HO-1, a key rate-limiting enzyme involved in the conversion of heme to iron, carbon monoxide (CO), and bilirubin [67] is known to be induced by environmental stimuli such as reactive oxygen species (ROS), cytokines, and endotoxin [68]. In the present study, both gene and protein expression of HO-1 were augmented in the BCO1/BCO2 DKO mice with the resultant development of hepatic steatosis. Consistent with our findings, Malaguarnera et al. reported induced HO-1 expression subsequent to disease progression from normal/simple steatosis to NASH [69]. A study by Jais et al. reported improvement to hepatic steatosis and TG levels in HO-1 KO mice fed a high-fat diet, suggesting that hepatic HO-1 was a potential predictor of disease severity [70]. The possible mechanism responsible for increases to HO-1 could result from reduced miR-122 and impaired FXR signaling in the BCO1/BCO2 DKO mice. Since the inhibition of miR-122 up-regulates HO-1 mRNA expression in hepatocytes [49], reduced miR-122 may lead to increased HO-1 levels in these mice. Consistent with our findings, the recent data by Wu et al. demonstrated that BCO2 KO mice exhibited higher oxidative stress with higher ROS levels, mitochondrial

dysfunction, and suppression of antioxidant enzymes including SOD2 and glutathione-disulfide reductase (GSR) [16]. These results support the hypothesis that the cooperative role of BCO1 and BCO2 enzymes includes the regulation of oxidative stress by regulating HO-1, SOD1, SOD2, GPX, and catalase by miR-122 (which is regulated by the FXR pathway) in the development of NAFLD.

In the present study, we cannot exclude the possibility that the functions of BCO1 and BCO2 are dependent, at least in part, on their ability to cleave carotenoids. Indeed, Shmarakov et al. [71] demonstrated that ablation of BCO1 induced BCO2 expression, and produced apo-carotenoids, which could lead to lipid accumulation in mouse livers. The present study demonstrated that ablation of both BCO1 and BCO2 resulted in the development of fatty liver in BCO1/BCO2 DKO mice. Interestingly, we did not observe the yellowish phenotype in the BCO1/BCO2 DKO mice. We detected only a small amount of lutein (1.22 nmol/g liver tissue) in the livers of BCO1/BCO2 DKO mice but none in WT mice. We did not observe any accumulation of other carotenoids or their metabolites in either WT or BCO1/BCO2 DKO mice. Since the source of lutein was possibly from wheat used in the manufacture of the chow diet, we analyzed carotenoid levels in the chow diet and lutein was the only carotenoid detected (10 mg/kg diet) and was at levels that were much lower than previously utilized (50 mg lutein/kg diet) [26]. Qiu et al. had previously reported that lutein supplementation ameliorated high fat diet-induced hepatic steatosis and increased the expression of hepatic SIRT1 and PPAR- α [72]. The chow diet used in this study contained sufficient vitamin A, and there was no significant difference in retinol and retinyl palmitate levels between the WT and BCO1/BCO2 DKO mice. Accordingly, it is unlikely that the development of NAFLD in the BCO1/2 DKO mice was due to any deficiency of vitamin A.

5. Conclusions

The current study provided strong experimental evidence that the ablation of both BCO1/BCO2 can lead to the development of NAFLD, thus suggesting that BCO1/BCO2 could play a significant role in maintaining normal hepatic lipid and cholesterol homeostasis, potentially through the regulation of the FXR/miR-34a/SIRT1 pathway. Our results furthered the growing body of evidence demonstrating that BCO1 and BCO2 have multiple roles in addition to their carotenoid cleavage activity. Further, our results emphasize the necessity of investigation of the relationship between genetic BCO1/BCO2 polymorphisms in humans and the metabolic diseases including NAFLD.

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Abbreviations

NAFLD	non-alcoholic fatty liver disease
BCO1	β -carotene-15, 15'-oxygenase
BCO2	β -carotene-9', 10'-oxygenase
DKO	double knock out
WT	wild type

FXR	farnesoid X receptor
SIRT1	sirtuin 1
SHP	small heterodimer partner
MiR	microRNA
TG	triglyceride
TC	total cholesterol
SCD1	stearoyl-CoA desaturase 1
ACC	acetyl-CoA carboxylase
CPT1	carnitine palmitoyltransferase1
PPARα	peroxisome proliferator-activated receptor alpha
CYP7A1	cholesterol 7 α -hydroxylase
CYP8B1	cholesterol 12 α -hydroxylase
HMG-CoAR	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HO-1	heme oxygenase-1
SOD1	superoxide dismutase1
SOD2	superoxide dismutase2
GPX	glutathione peroxidase
SNP	single nucleotide polymorphism
HPLC	high performance liquid chromatography

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.abb.2018.07.007>.

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Dietary β -Cryptoxanthin Inhibits High-Refined Carbohydrate Diet-Induced Fatty Liver via Differential Protective Mechanisms Depending on Carotenoid Cleavage Enzymes in Male Mice

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ABSTRACT

Background: β -Cryptoxanthin (BCX), a provitamin A carotenoid shown to protect against nonalcoholic fatty liver disease (NAFLD), can be cleaved by β -carotene-15,15'-oxygenase (BCO1) to generate vitamin A, and by β -carotene-9',10'-oxygenase (BCO2) to produce bioactive apo-carotenoids. BCO1/BCO2 polymorphisms have been associated with variations in plasma carotenoid amounts in both humans and animals.

Objectives: We investigated whether BCX feeding inhibits high refined-carbohydrate diet (HRCD)-induced NAFLD, dependent or independent of BCO1/BCO2.

Methods: Six-week-old male wild-type (WT) and BCO1^{-/-}/BCO2^{-/-} double knockout (DKO) mice were randomly fed HRCD (66.5% of energy from carbohydrate) with or without BCX (10 mg/kg diet) for 24 wk. Pathological and biochemical variables were analyzed in the liver and mesenteric adipose tissues (MATs). Data were analyzed by 2-factor ANOVA.

Results: Compared to their respective HRCD controls, BCX reduced hepatic steatosis severity by 33–43% and hepatic total cholesterol by 43–70% in both WT and DKO mice ($P < 0.01$). Hepatic concentrations of BCX, but not retinol and retinyl palmitate, were 33-fold higher in DKO mice than in WT mice ($P < 0.001$). BCX feeding increased the hepatic fatty acid oxidation protein peroxisome proliferator-activated receptor- α , and the cholesterol efflux gene ATP-binding cassette transporter5, and suppressed the lipogenesis gene acetyl-CoA carboxylase 1 (*Acc1*) in the MAT of WT mice but not DKO mice ($P < 0.05$). BCX feeding decreased the hepatic lipogenesis proteins ACC and stearoyl-CoA desaturase-1 (3-fold and 5-fold) and the cholesterol synthesis genes 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and HMG-CoA synthase 1 (2.7-fold and 1.8-fold) and increased the cholesterol catabolism gene cholesterol 7 α -hydroxylase (1.9-fold) in the DKO but not WT mice ($P < 0.05$). BCX feeding increased hepatic protein sirtuin1 (2.5-fold) and AMP-activated protein kinase (9-fold) and decreased hepatic farnesoid X receptor protein (80%) and the inflammatory cytokine gene *Il6* (6-fold) in the MAT of DKO mice but not WT mice ($P < 0.05$).

Conclusion: BCX feeding mitigates HRCD-induced NAFLD in both WT and DKO mice through different mechanisms in the liver-MAT axis, depending on the presence or absence of BCO1/BCO2. *J Nutr* 2019;149:1553–1564.

Keywords: NAFLD, high-refined carbohydrate diet, β -cryptoxanthin, carotenoid cleavage enzymes, sirtuin 1, nuclear receptors

Introduction

Nonalcoholic fatty liver disease (NAFLD) affects 30%, 20%, and 3–10% of adult men, women, and children, respectively, in the USA, and its incidence is rapidly accelerating to cause a tidal wave of disability, illness, and death (1). The prevalence of

NAFLD is 80–90% in obese adults, 30–50% in patients with diabetes, and upwards of 40–70% among obese children (2). Because limited treatment and pharmacological options exist for NAFLD, there is an urgent need to determine the efficacy of dietary intervention in prevention of NAFLD.

Accumulating evidence from epidemiological and animal studies has shown that high consumption of a high-refined carbohydrate diet (HRCd), including high fructose and high sugar, is intimately associated with the development and severity of NAFLD (3–5). This is mainly a result of the deleterious effects of fructose, which can be delivered directly to the liver via the portal vein, thus contributing to liver injury (6). We previously found that replacing a high-fat diet with an HRCd (including both maltodextrin and sucrose disaccharide, which can be broken down to the monosaccharides glucose and fructose) significantly exacerbated hepatic steatosis (5). We also observed that HRCd-fed mice exhibited more severe hepatic steatosis than high-fat diet-fed mice, which was associated with a significant upregulation of *de novo* lipogenesis (DNL) genes and proteins (7). These findings suggest that in addition to reducing HRCd consumption, another promising strategy may be to identify bioactive dietary components that effectively target the development of HRCd-induced NAFLD.

β -Cryptoxanthin (BCX), a provitamin A xanthophyll carotenoid, is found at high concentrations in sweet red peppers (*Capsicum annuum* L.) and their dietary product, paprika, and at lower concentrations in butternut squash, pumpkins, tangerines, oranges, and papayas (8, 9). Several epidemiological studies have shown that high serum concentrations of BCX are inversely associated with the risk of certain types of cancers (10, 11), type 2 diabetes (12), and a reduction in BMI (13), liver disorders (14), and NAFLD (15). Several clinical intervention trials have shown that consumption of a BCX beverage increased adiponectin concentrations (16), and decreased BMI (17), visceral fat accumulation (18), serum cholesterol concentration (19), and amounts of biomarkers of oxidative stress and inflammation in NAFLD patients (20). Previously, BCX supplementation was shown to suppress insulin resistance and nonalcoholic steatohepatitis (21, 22) and to reduce inflammatory cytokines in adipose tissues of obese mice (23); however, the effects of BCX feeding on HRCd-induced NAFLD development are unknown. Importantly, BCX is preferentially cleaved by β -carotene-15,15'-oxygenase (BCO1) and β -carotene-9',10'-oxygenase (BCO2) to produce bioactive metabolite products including vitamin A (8, 9). However, whether the protective role of BCX is dependent on BCO1/BCO2 is unclear, and the presence of single nucleotide

polymorphisms (SNPs) in BCO1/BCO2 enzymes in humans makes this a pertinent question. It has been shown that SNPs in BCO1/BCO2 enzymes are associated with carotenoid status (24), age-related eye diseases (25), and increased IL-18 concentrations (26).

Recently, we demonstrated that ablation of both BCO1 and BCO2 from mice fed a standard unpurified diet induced NAFLD potentially through impairment of the farnesoid X receptor (FXR) and sirtuin 1 (SIRT1) pathway (27). FXR, a metabolic nuclear receptor, plays pivotal roles in the maintenance of bile acid homeostasis and lipid and cholesterol metabolism (28, 29). SIRT1, a NAD⁺-dependent protein/histone-deacetylase, has been associated with development of NAFLD (30). SIRT1 is known to be a metabolic regulator of fatty acid synthesis (31), enhancer of fatty acid β -oxidation (32), and an inhibitor of inflammation (33), oxidative stress (34), and adipocyte differentiation (35). Ablation of SIRT1 aggravated liver steatosis by facilitating the release of free fatty acids (FFAs) from mesenteric adipose tissue (MAT) (36). The AMP-activated protein kinase (AMPK) is a master regulator of energy homeostasis and has health implications for treating insulin resistance, type 2 diabetes, and NAFLD (37). AMPK activates SIRT1 by increasing the NAD⁺/NADH ratio (38, 39) and regulating a variety of metabolic pathways including hepatic lipid accumulation (39). Although we investigated the potential role of BCX in activating SIRT1 using a mouse model of lung cancer (40), the effects of BCX on the FXR/SIRT1/AMPK pathway remain largely obscure. In addition, it remains unexplored whether BCX regulates the FXR/SIRT1/AMPK pathway in HRCd-induced NAFLD depending on BCO1/BCO2 activity.

In the present study, we investigated the role of BCX in the development of HRCd-induced NAFLD using BCO1/BCO2 double knockout (DKO) mice lacking the carotenoid cleavage enzymes and the respective wild-type (WT) mice. We also investigated multiple molecular mechanisms and the potential involvement of the FXR/SIRT1/AMPK pathway on the BCX role in the liver-MAT axis.

Methods

Animals, diets, and study design

Male WT and BCO1^{-/-}/BCO2^{-/-} double knockout (DKO) mice (BCO1/BCO2 DKO), congenic on the C57BL/6 J genetic background, were generated as previously described (27). Because we previously observed a dramatic male predominance in the incidence of steatosis with no steatosis detected in female mice (41), only male mice were used in the present study. We previously demonstrated that WT mice fed a standard unpurified diet did not develop NAFLD, whereas DKO mice fed a standard unpurified diet developed very mild NAFLD (hepatic steatosis score of 1.0) (27). Moreover, we showed that mice fed HRCd for 24 wk showed a greater severity of NAFLD (pathologic hepatic steatosis score of 3.0) than high-fat diet-fed C57BL/6 J WT mice (7). Because our aim in the present study was to determine whether dietary BCX inhibits NAFLD in either the absence or presence of carotenoid cleavage enzymes, all WT and DKO mice were fed the same semipurified HRCd diet used previously (7) to induce NAFLD based on assessment by histopathologic score analyses. All experimental procedures were performed in accordance with the animal protocols approved by the Tufts University Institutional Animal Care and Use Committee. After 2 wk of acclimation, 6 wk-old WT and DKO mice were randomly assigned to receive either HRCd (66.5% of calories were from carbohydrate consisting of maltodextrin and sucrose) that contained sufficient vitamin A without carotenoids, as described in our previous study (7), or HRCd supplemented with BCX (daily dose of 10

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Supplemental Table 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

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Abbreviations used: ABCG, ATP-binding cassette transporter; ACC, acetyl-CoA carboxylase; ACOX1, acyl-CoA oxidase 1; AMPK, AMP-activated protein kinase; BCO1, β -carotene-15,15'-oxygenase; BCO2, β -carotene-9',10'-oxygenase; BCX, β -cryptoxanthin; CYP7A1, cholesterol 7 α -hydroxylase; DKO, double knockout; DNL, *de novo* lipogenesis; FAS, fatty acid synthase; FFA, free fatty acid; FXR, farnesoid X receptor; HRCd, high-refined carbohydrate diet; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HMGSL, 3-hydroxy-3-methyl-glutaryl-coenzyme A synthase 1; MAT, mesenteric adipose tissue; *mir34a*, microRNA-34a; NAFLD, nonalcoholic fatty liver disease; PPARA, peroxisome proliferator-activated receptor- α ; SCD1, stearoyl-CoA desaturase-1; SHP, small heterodimer partner; SIRT1, sirtuin 1; SNP, single nucleotide polymorphism; SREBF2, sterol regulatory element binding transcription factor 2; TC, total cholesterol; TG, triglyceride; WT, wild-type.

mg/kg diet). All animals were fed the experimental diets ad libitum with free access to water for 24 wk as previously described (7). All mice were housed and maintained under environmental conditions of $22 \pm 2^\circ\text{C}$, $40 \pm 5\%$ relative humidity, and 12-h light/12-h dark cycle. During the experimental period, body weights of all animals were measured twice weekly. At the conclusion of the dietary intervention, the animals were killed by terminal exsanguination (for serum) under IsothesiaTM (Henry Schein) anesthesia without overnight fasting. Liver and MAT were excised and weighed. The central left lobe of liver was collected, and fixed in 10% buffered formalin solution (Thermo Fisher Scientific), and the remaining portions of liver were snap-frozen and stored at -80°C for further analysis.

BCX supplementation

The BCX dosage (10 mg/kg diet) was established from our previous work as the dose reported to reduce murine lung carcinogenesis (42). The BCX concentrate (MJ-71-01) was provided by Dr. Vijaya Juturu (Omni Active Health Technologies Pvt. Ltd). The BCX concentrate was extracted/purified from paprika and suspended in oil, as previously described (23). We confirmed that the BCX concentrate contained 12.5% BCX and no other carotenoids were detectable by our HPLC analysis. We mixed 80 mg of BCX concentrate into each 1 kg of HRCD powder diet (Bio-Serv) under red light to obtain a final concentration of 10 mg BCX/kg diet. The diet was stored at -20°C . The diets were changed twice a day during feeding. We also conducted a stability test of BCX in diet and observed that BCX remained stable in the diet for 3–4 d. The dose selected for use in this study is equivalent to a daily human consumption of 0.87 mg, which is comparable to consuming 85.0–113 g of sweet red pepper daily (42), and thus this dose of BCX is considered physiologically relevant.

Liver histopathology evaluation

Five-micrometer sections of formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin to determine the liver steatosis score as described previously (27). Sections were examined under a light microscope by 2 independent investigators who were blinded to the treatment groups, as described previously (27).

RT-PCR and qRT-PCR

Total RNA extraction from liver and MAT samples and subsequent qRT-PCR were performed as previously described (27). Quantitative PCR was performed on an Applied Biosystems 7000 machine with FastStart Universal SYBR Green Master Mix (Roche). Selected primers are described in Supplemental Table 1 and β -actin was used as an internal control.

MicroRNA quantification by real-time qRT-PCR analysis

Total RNA extraction and subsequent cDNA synthesis for microRNA-34a (*mir34a*) analysis were performed as previously described (27). Quantitative PCR was performed on an Applied Biosystems 7000 machine with FastStart Universal SYBR Green Master Mix (Roche). U6 snRNA was used as an internal control.

Western blot analyses

Western blot analyses were performed in the liver tissues from all animals as previously described (27). The following antibodies were used: SIRT1 (Santa Cruz); FXR (GeneTex), peroxisome proliferator-activated receptor- α (PPARA; Abcam); SCD1, ACC, total AMPK (Cell Signaling), and β -actin (Sigma-Aldrich). The densities of specific relative bands were quantified by densitometric scanning using a model GS-710 Calibrated Imaging Densitometer (Bio-Rad); β -actin was used to confirm equal loading.

Hepatic triglyceride, total cholesterol, and plasma FFA analysis

Hepatic triglyceride (TG), total cholesterol (TC), and plasma FFA concentrations were measured using a commercial TG colorimetric assay kit (Cayman), TC assay kit (Cell Biolabs Inc.), and FFA

quantification assay kit (Abcam), respectively. All procedures were performed according to the protocol obtained from the manufacturer.

HPLC analysis

Liver samples for HPLC analysis were prepared as previously described (27). The amounts of retinol, retinyl palmitate, and BCX were analyzed in the liver tissue using gradient reversed phase HPLC consisting of a Waters 2996 photodiode array detector (Waters), a Waters 2695 separation module and a reverse-phase C18 column (4.6×250 mm, $5 \mu\text{m}$) with a flow rate of 1.00 mL/min. Retinol, retinyl palmitate, and BCX concentrations were calculated by determining the peak areas in the chromatograms calibrated against known amounts of standard. All procedures were conducted under red light.

Statistical analysis

All statistical analyses were performed using GraphPad-Prism 7.0 software (GraphPad Software). Results are presented as the means $\pm$ SEMs or medians. Data were tested for normal distribution by Shapiro-Wilk normality test. Two-factor ANOVA was performed to assess BCX, strain, and BCX-strain interactions among groups. One-factor ANOVA followed by Tukey's post hoc analysis was conducted when significant effects were detected using a 2-factor ANOVA. Nonparametric Kruskal-Wallis test with Benjamini-Hochberg false discovery rate for multiple testing corrections was used to compare hepatic scores, and correlations were tested by Spearman's test. To determine differences in variances for ANOVAs, Bartlett's test was performed. For hepatic BCX concentrations, comparisons between the 2 genotypes were analyzed by Student's *t* test. Differences among groups were regarded as statistically different when $P < 0.05$.

Results

BCX suppresses hepatic lipid accumulation in both WT and DKO mice

Mean final body weight (g) and liver weight/body weight did not differ significantly among all groups (Table 1). MAT weight/body weight were significantly lower in the HRCD and HRCD + BCX groups of the DKO mice than in the WT-HRCD group ($P < 0.05$), but there were no significant differences between the HRCD and HRCD + BCX groups irrespective of genotype (Table 1). Histological analyses demonstrated that HRCD induced pronounced hepatic steatosis in both WT and DKO mice (scores 3.0 and 3.5, respectively). However, BCX intervention significantly suppressed hepatic steatosis in both genotypes (score 2.0) when compared to that in their HRCD littermates ($P < 0.01$ for both; Table 1). Histopathological examination revealed that, irrespective of genotype, the presence of large and small fat droplets was markedly reduced by BCX in mice compared to that in their HRCD littermates (Figure 1). BCX feeding significantly reduced hepatic TC content by 43% and 70%, respectively, in the WT and DKO mice when compared to that in their HRCD littermates ($P < 0.001$ for both; Table 1). Furthermore, BCX feeding significantly decreased hepatic TG (36%) in the DKO mice when compared to that in their HRCD littermates ($P < 0.05$; Table 1). In both genotypes, hepatic TG and TC were correlated with the hepatic steatosis score ($\rho = 0.36, 0.72$; $P = 0.0193, <0.0001$, respectively). Together, these results indicate that BCX exerted ameliorative effects on HRCD-induced hepatic steatosis in both WT and DKO mice.

Retinol, retinyl palmitate, and BCX

Hepatic retinol and retinyl palmitate amounts did not significantly differ among all experimental groups (Table 1). BCX was not detected in the HRCD groups without BCX feeding

TABLE 1 Primary outcomes of WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX¹

Experimental group	WT		BCO1/BCO2 DKO		P values for 2-factor ANOVA		
	HRCD	HRCD + BCX	HRCD	HRCD + BCX	BCX effect	Strain effect	BCX-strain interaction
Number of mice	15	15	15	15			
Final body weight, g	46.0 ± 0.91	46.6 ± 1.07	46.2 ± 0.84	46.8 ± 1.00	0.52	0.82	0.96
Liver weight, g	2.89 ± 0.27	2.34 ± 0.16	2.73 ± 0.17	2.91 ± 0.21	0.38	0.34	0.08
Liver weight/body weight, %	6.23 ± 0.48	5.06 ± 0.34	5.85 ± 0.31	6.14 ± 0.37	0.35	0.25	0.06
Mesenteric fat weight, g	0.98 ± 0.09	0.88 ± 0.04	0.78 ± 0.17	0.77 ± 0.04	0.36	<0.05	0.46
Mesenteric fat weight/body weight, %	2.11 ± 0.18 ^a	1.92 ± 0.08 ^{ab}	1.69 ± 0.08 ^b	1.65 ± 0.09 ^b	0.25	<0.01	0.41
Hepatic steatosis ² incidence, % of mice	93% (14/15)	93% (14/15)	100% (15/15)	80% (12/15)	—	—	—
Hepatic steatosis score, median (range)	3.0 (0–4) ^a	2.0 (0–3) ^b	3.5 (1–4) ^a	2.0 (0–3) ^b	—	—	—
Hepatic TG, mg/g	19.0 ± 1.26 ^{ab}	15.6 ± 1.16 ^{bc}	20.8 ± 1.72 ^a	13.4 ± 1.19 ^c	<0.05	0.36	0.23
Hepatic TC, mg/g	97.5 ± 11.5 ^a	55.7 ± 6.26 ^b	120 ± 8.45 ^a	51.0 ± 7.08 ^b	<0.001	0.38	0.12
Plasma FFAs, μ M	159 ± 2.23 ^a	162 ± 4.87 ^a	144 ± 6.46 ^{ab}	127 ± 4.86 ^b	0.18	<0.001	0.06
Hepatic retinol, nmol/g	6.61 ± 1.22	6.08 ± 1.24	5.87 ± 1.33	5.25 ± 0.79	0.65	0.53	0.97
Hepatic retinyl palmitate, mmol/g	0.28 ± 0.08	0.40 ± 0.09	0.28 ± 0.08	0.34 ± 0.24	0.29	0.73	0.56
Hepatic BCX, nmol/g	N/D	0.50 ± 0.04	N/D	16.7 ± 3.84 ³	<0.001	<0.001	<0.001

¹Values are presented as means ± SEMs, $n = 10$ – 15 (final body weight, liver weight, mesenteric fat weight, TG, TC, and FFA) or 8 (hepatic retinol, retinyl palmitate, and BCX) or medians (ranges), $n = 15$ (hepatic steatosis score). Means without a common letter differ ($P < 0.05$). BCO1/BCO2 DKO, β -carotene-15,15'-oxygenase/ β -carotene-9',10'-oxygenase double knockout; FFA, free fatty acid; HRCD, high-refined carbohydrate diet; HRCD + BCX, high-refined carbohydrate diet supplemented with β -cryptoxanthin; N/D, not detectable; TC, total cholesterol; TG, triglycerides; WT, wild-type.

²Hepatic steatosis is defined as intrahepatic lipid accumulation >5% of the liver weight.

³Different from WT-HRCD + BCX group, $P < 0.05$.

in WT and DKO mice (Table 1). BCX amounts in the liver were significantly higher (33-fold) in the DKO-HRCD + BCX group than in the WT-HRCD + BCX group ($P < 0.001$; Table 1). We did not detect any other carotenoids or apo-carotenoids (such as β -apo-10'-carotenal or 3-hydroxy- β -apo-10'-carotenal) in the livers of any of the groups.

Differential actions of BCX in lipid metabolism in WT and DKO mice

The imbalance between lipogenesis and fatty acid β -oxidation can result in hepatic lipid accumulation in the liver. In DKO mice, but not in WT mice, BCX significantly lowered the mRNA levels of lipogenesis markers including fatty acid synthase

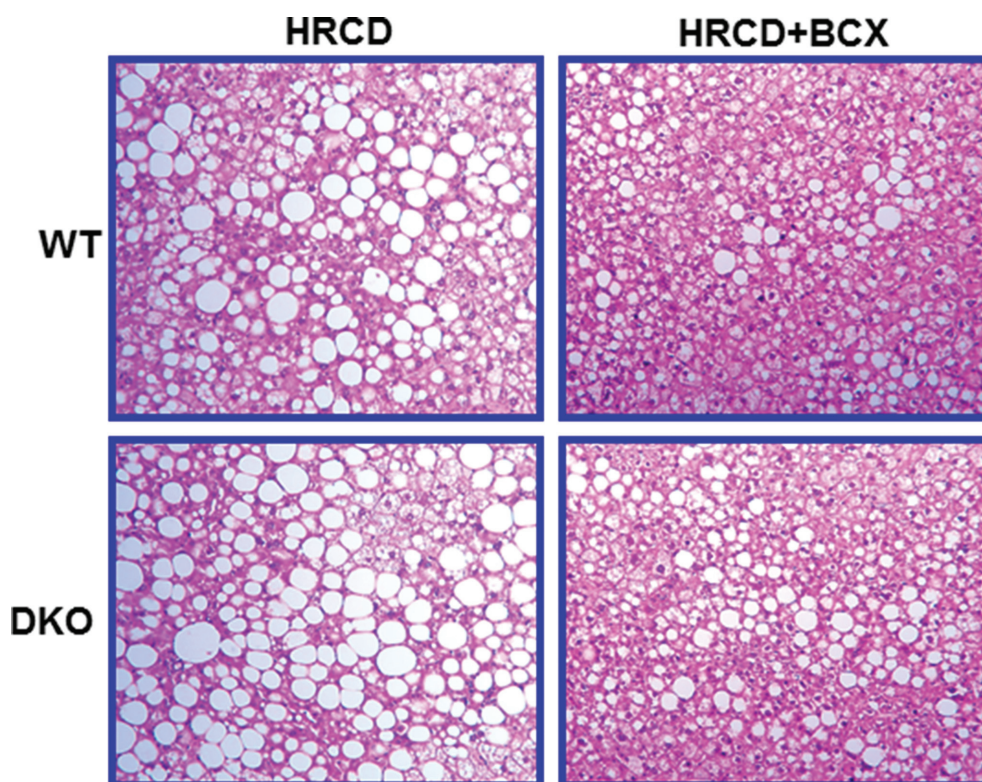


FIGURE 1 Histopathological analysis of the livers of WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Hematoxylin and eosin-stained slides were imaged microscopically and photographed at 100 $\times$. BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; WT, wild-type.

(*Fas*) and acetyl-CoA carboxylase 2 (*Acc2*) by 40% and 42%, respectively ($P < 0.01$ and $P < 0.01$, respectively), compared to those in the HRCD group (Figure 2A and C). Moreover, the protein levels of lipogenesis markers including ACC and stearoyl-CoA desaturase-1 (SCD1) were significantly downregulated (3-fold and 5-fold, respectively) by BCX feeding in DKO mice, but not in WT mice, as compared to levels in their HRCD littermates ($P < 0.05$ for both; Figure 2F). Interestingly, *Fas* mRNA and SCD1 protein expressions were significantly higher (30% and 6-fold) in the DKO-HRCD group, compared with those in the WT-HRCD group ($P < 0.05$ for both; Figure 2A and F). In WT mice, but not in DKO mice, BCX significantly increased gene expression levels of fatty acid β -oxidation markers, including peroxisome proliferator-activated receptor- α (*Ppara*) and acyl-CoA oxidase 1 (*Acox1*), (26% for both; $P < 0.05$) compared to those in the HRCD group (Figure 2D, E). PPARA protein levels were also significantly induced (2.5-fold) by BCX feeding in WT mice, but not in DKO mice, compared to those in their HRCD littermates ($P < 0.05$; Figure 2F). These results suggest that BCX impacts lipid metabolism differently depending on the presence or absence of carotenoid cleavage enzymes. BCX suppressed lipogenesis in DKO mice and increased fatty acid β -oxidation in WT mice, which can contribute to the mitigation of liver steatosis in both genotypes.

Differential actions of BCX in cholesterol metabolism in WT and DKO mice

Hepatic cholesterol homeostasis is maintained by coordinated regulation of cholesterol biosynthesis and uptake, cholesterol conversion to bile acids, and cholesterol efflux. In DKO mice, but not in WT mice, BCX significantly increased (1.9-fold) the mRNA expression levels of hepatic cholesterol 7 α -hydroxylase (*Cyp7a1*), a rate-limiting enzyme for bile acid synthesis, when compared to the levels in HRCD mice (Figure 3A). Notably, BCX significantly reduced mRNA levels of 3-hydroxy-3-methyl-glutaryl-coenzymeA reductase (*Hmgcr*) and 3-hydroxy-3-methyl-glutaryl-coenzyme A synthase 1 (*Hmgs1*) in DKO mice by 2.7-fold and 1.8-fold, respectively, when compared to the levels in their HRCD littermates ($P < 0.001$ and $P < 0.05$, respectively; Figure 3C and D). In WT mice, but not in DKO mice, BCX significantly upregulated (2.5-fold) the mRNA levels of the cholesterol transporter, ATP-binding cassette transporter 5 (*Abcg5*), when compared to the levels in their HRCD littermates ($P < 0.05$; Figure 3E). Similar changes were observed in mRNA levels of *Abcg8*, but it was not significant (Figure 3F). Gene expression of LDL-receptor (*Ldlr*), cholesterol uptake marker, was not significantly different among groups (data not shown). HRCD-fed DKO mice had significant increases in cholesterol synthesis biomarkers including sterol regulatory element-binding transcription factor-2 (*Srebf2*) and *Hmgcr* compared to HRCD-fed WT mice (1.5- and 3.0-fold respectively) ($P < 0.001$ and $P < 0.0001$, respectively; Figure 3B and C). These data suggest that BCX contributes to reducing hepatic TC concentration by promoting cholesterol catabolism and reducing cholesterol biosynthesis in DKO mice while increasing cholesterol efflux in WT mice.

Differential actions of BCX on nuclear receptors in WT and DKO mice

In DKO mice, BCX significantly suppressed both gene and protein expression of FXR (69% and 80%, respectively) when

compared to the expression in their HRCD littermates ($P < 0.01$ for both; Figure 4A and B). In WT mice, however, BCX resulted in significant elevation in protein concentrations of FXR (2.0-fold) and mRNA levels of its downstream target, small heterodimer partner (*Shp*) (50%), compared to their expression in their HRCD littermates ($P < 0.001$ and $P < 0.05$, respectively; Figure 4B and C). These data suggest that BCX regulates the nuclear receptors differently, depending on BCO1/BCO2.

Intact BCX activates the SIRT1/AMPK pathway in the livers of DKO mice

Although no significant differences in *Sirt1* mRNA expression were observed among all groups (Figure 5A), BCX significantly upregulated SIRT1 protein (2.5-fold) in DKO mice, but not in WT mice, when compared to the HRCD group ($P < 0.05$; Figure 5B). Moreover, BCX significantly induced AMPK protein (9-fold) in DKO mice, but not in WT mice, when compared to their HRCD littermates ($P < 0.05$; Figure 5B). The levels of *mir34a*, a direct inhibitor of SIRT1, were not significantly different among groups (Figure 5C). These data suggest that intact BCX ameliorated hepatic steatosis potentially by upregulating the SIRT1/AMPK pathway, especially in DKO mice.

Intact BCX increases fatty acid β -oxidation and suppresses *Il6* and *Il4* potentially by elevating *Sirt1* in the MAT of DKO mice

In WT mice, but not in DKO mice, BCX significantly reduced *Acc1* mRNA levels (70%) when compared to levels in the HRCD group ($P < 0.05$; Figure 6A). Of note, BCX significantly induced *Acox1* mRNA levels (2.6-fold) in DKO mice, but not in WT mice, when compared to the HRCD group ($P < 0.05$; Figure 6B). Interestingly, proinflammatory cytokine, *Il6* mRNA levels were significantly induced by HRCD and attenuated by BCX (6-fold) in DKO mice ($P < 0.01$; Figure 6C). Moreover, an anti-inflammatory cytokine, *Il4* mRNA levels were significantly increased by HRCD and inhibited by BCX (5.5-fold) in DKO mice ($P < 0.01$; Figure 6D). Notably, we observed that BCX significantly increased mRNA levels of *Sirt1* by ~2.5-fold in DKO mice when compared to the levels in the HRCD group ($P < 0.001$; Figure 6E). These data suggest that BCX enhanced fatty acid β -oxidation and suppressed the *Il6* and *Il4*, potentially by elevating *Sirt1* in DKO mice.

Discussion

In the present study, we provided evidence that dietary BCX effectively prevents development of HRCD-induced NAFLD in both BCO1/BCO2 DKO mice and the corresponding WT control mice. Notably, our data suggest that the mechanisms involved are different in WT and DKO mice (Figure 7), indicating that the BCX molecule, when not cleaved, exerts independent functions. The BCX-induced mitigation of hepatic steatosis in the absence of BCO1/BCO2 suggests that intact dietary BCX plays a role in prevention of NAFLD. There were no significant differences in the hepatic amounts of retinol or retinyl palmitate among the groups, implying that intact BCX has a unique role in prevention of HRCD-induced NAFLD, without generating vitamin A. Importantly, we observed that the protective effects of BCX against

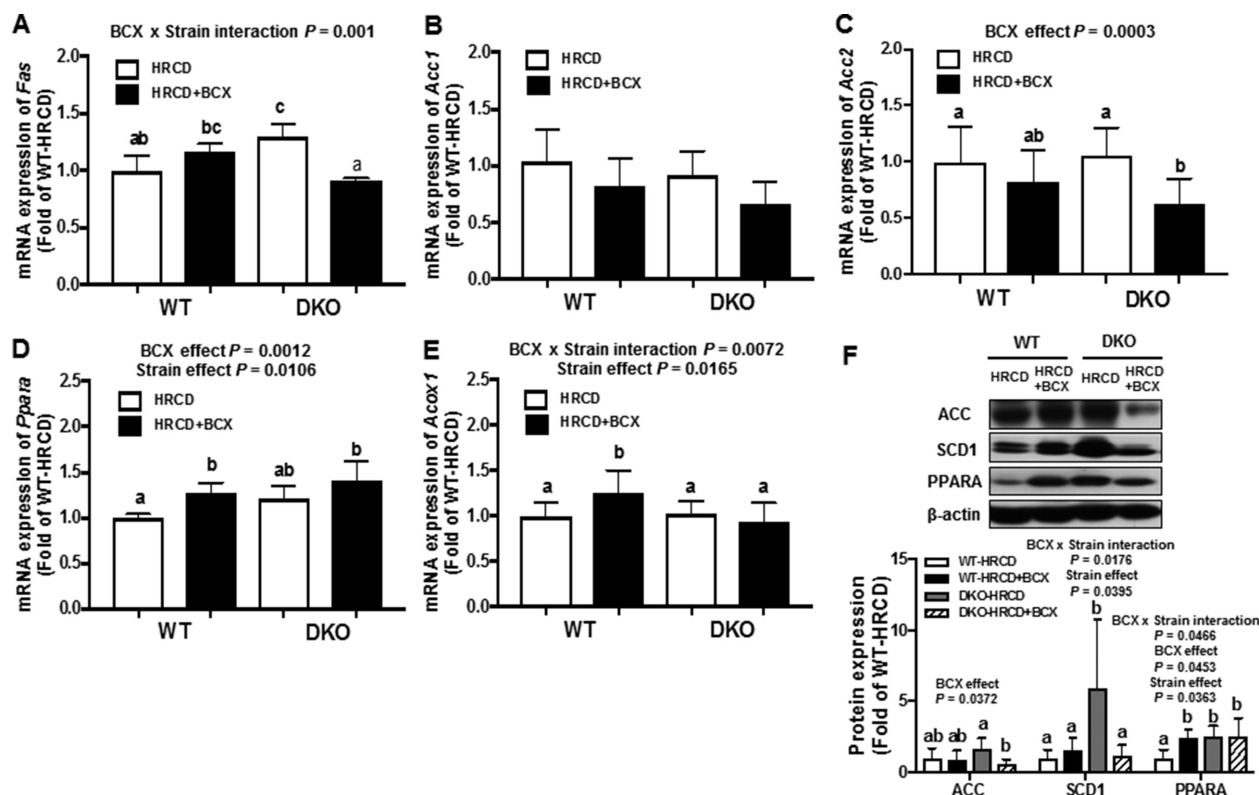


FIGURE 2 Effects of BCX on hepatic lipid metabolism markers in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in the mRNA expression of fatty acid synthesis markers (A) *Fas*, (B) *Acc1*, and (C) *Acc2* and fatty acid β -oxidation markers (D) *Ppara*, (E) *Acox1*, and (F) ACC, SCD1, and PPAR α proteins quantified via real-time PCR or Western blot in the livers of WT mice and DKO mice fed HRCD with or without BCX. β -Actin was used as a loading control. Values are means $\pm$ SEMs, $n = 10$ –12 except (F) where $n = 5$ or 6. Means without a common letter differ, $P < 0.05$. Acc, acyl CoA carboxylase; *Acox1*, acyl CoA oxidase 1; BCX, β -cryptoxanthin; DKO, double knockout; *Fas*, fatty acyl synthase; HRCD, high-refined carbohydrate diet; *Ppara*, peroxisome proliferator activated receptor- α ; SCD1, stearoyl-CoA desaturase-1; WT, wild-type.

NAFLD are achieved through different molecular mechanisms in the liver-MAT axis and depend on carotenoid cleavage enzymes.

We demonstrated that BCX effectively suppressed hepatic TG in DKO mice, partly through the suppression of DNL. Although a large proportion of hepatic TG is derived from plasma FFAs (43), DNL is the major contributor to synthesis of TG in NAFLD (44). Thus, the BCX-mediated reduction in TG in DKO mice potentially resulted from suppression of hepatic DNL, which was associated with an increase in SIRT1 and AMPK protein by BCX feeding in DKO mice (Figure 7B). This increase in SIRT1 and AMPK protein has been reported to result in inhibition of FAS and ACC expression and prevention of lipid accumulation in the liver (39), indicating that intact BCX-mediated activation of the SIRT1/AMPK pathway possibly contributes to inhibition of lipogenesis markers in the livers of DKO mice. Unlike in DKO mice, the induction of SIRT1 and anti-lipogenic effects of BCX were not observed in WT mice. In addition, in WT mice, the amounts of the fatty acid β -oxidation markers PPAR α and ACOX1 were increased, possibly as a result of upregulation of FXR and subsequent induction of hepatic nuclear SHP (45) (Figure 7A). In line with our results, several studies have reported that FXR/SHP activation improves hyperlipidemia (46) and liver steatosis (47, 48). Overall, our results suggest that the effects of BCX on lipogenesis and fatty

acid β -oxidation depend on the intact BCX molecule or its cleavage derivatives, respectively.

Animal and clinical studies have shown that BCX effectively reduces plasma and hepatic cholesterol concentrations (19, 21, 23, 49). Interestingly, we observed that BCX mitigated hepatic TC accumulation in both DKO and WT mice. It is possible that a combination of increased cholesterol bioconversion into bile acids and decreased cholesterol biosynthesis by BCX contributes to the mitigation of hepatic TC accumulation in DKO mice. This notion was supported by our observation that BCX markedly increased *Cyp7a1* mRNA expression in DKO mice, possibly resulting from FXR suppression, which reportedly reduces hypercholesterolemia by inducing CYP7A1 (50–52). BCX has also been reported to induce the expression of CYP27A1, an enzyme involved in conversion of excess cholesterol into 27-hydroxycholesterol (53). It is thus conceivable that intact BCX suppressed FXR and subsequently activated CYP7A1, thereby increasing hepatic TC catabolism and consequently reducing hepatic TC in DKO mice (Figure 7B). Given that AMPK has been shown to directly inhibit FXR transcriptional activity (54), we speculate that the inhibition of FXR by BCX in DKO mice might be caused by AMPK activation. In addition to the beneficial effects of FXR activation, accumulating evidence also suggests that FXR inactivation is beneficial for ameliorating dyslipidemia, especially hypercholesterolemia (51,

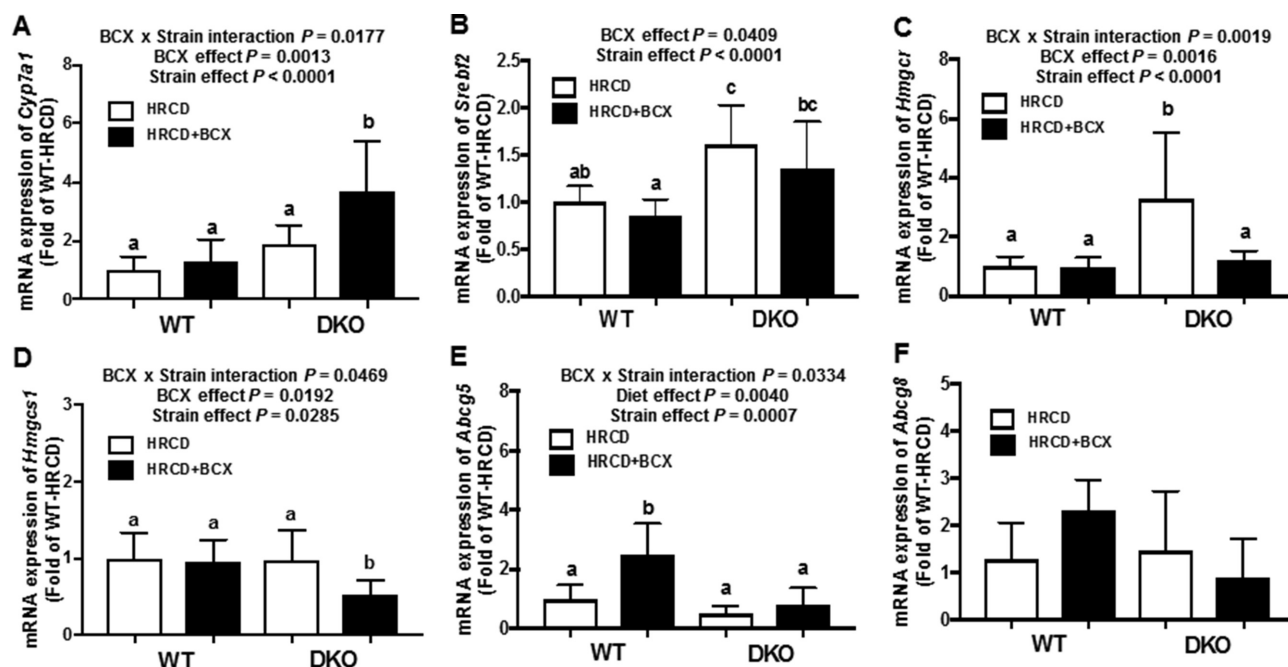


FIGURE 3 Effects of BCX on hepatic cholesterol metabolism markers in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in the mRNA expression of bile-acid synthesis markers (A) *Cyp7a1*, cholesterol synthesis markers, (B) *Srebf2*, (C) *Hmgcr*, and (D) *Hmgcs1* and cholesterol efflux markers, (E) *Abcg5*, and (F) *Abcg8* quantified via real-time PCR in the livers of WT mice and DKO mice fed HRCD with or without BCX. β -Actin was used as a loading control. Values are means $\pm$ SEMs, $n = 10-12$. Means without a common letter differ, $P < 0.05$. *Abcg5*, ATP-binding cassette transporter 5; *Abcg8*, ATP-binding cassette transporter 8; BCX, β -cryptoxanthin; *Cyp7a1*, cholesterol 7 α -hydroxylase; DKO, double knockout; *Hmgcr*, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, *Hmgcs1*, HMG-CoA synthase 1; HRCD, high-refined carbohydrate diet; *Srebf2*, sterol regulatory element-binding protein-2; WT, wild-type.

52). Our results align with those of the recent study by Kim et al. showing that deletion of FXR improved hepatic lipid accumulation by upregulating the *Cyp7a1* gene in aged mice (55). Moreover, we demonstrated that intact BCX effectively inactivates the expression of cholesterol biosynthesis markers (including *Hmgcr* and *Hmgcs1*) in DKO mice (56). This probably occurs through upregulation of SIRT1 and AMPK induced by BCX feeding. Inhibition of SIRT1 and AMPK in NAFLD is reportedly associated with high cholesterol synthesis via downregulation of inhibition of HMGCR phosphorylation (56, 57). Unlike in DKO mice, in WT mice, cholesterol efflux was a major contributor to the BCX-mediated decrease in hepatic TC, which may have been affected by upregulation of FXR and SHP expression (58) (Figure 7A). Together, our results suggest that BCX regulates cholesterol metabolism through differential regulation of the FXR/SIRT1/AMPK pathway in the liver, depending on BCO1/BCO2. However, the mechanisms underlying FXR/SIRT1/AMPK regulation by BCX, depending on the presence or absence of BCO1/BCO2, remain elusive and require further research.

In the current study, the MAT/body weight ratio was slightly but significantly lower in the DKO-HRCD mice than in the WT-HRCD mice. Hepatic steatosis score, hepatic steatosis incidence, hepatic TG, and hepatic TC were marginally but not significantly higher in DKO-HRCD than in WT-HRCD mice. These metabolic perturbations may result from the deficiency of the BCO2 enzyme as reported by Ford et al. who observed that BCO2 KO mice exhibited significantly lower visceral adiposity and hepatic enlargement with lipid accumulation, suggesting that BCO2 may have specific roles in regulation of lipid

metabolic homeostasis between liver and adipose tissue (59). Thus, it is plausible that reduction of MAT in DKO-HRCD mice, relative to the WT-HRCD group, occurred because of the ablation of BCO2. Consistent with this hypothesis, we found that the lipogenesis marker (*Acc1*) was significantly lowered in the MAT, whereas the lipogenesis markers (*Fas* and *SCD1*) and the cholesterol synthesis markers (*Srebf2* and *Hmgcr*) were significantly higher in the liver in the DKO-HRCD group than in the WT-HRCD group.

MAT is regarded as potentially harmful because it contains higher amounts of the proinflammatory cytokine IL-6 than other adipose tissues (60), which plays a crucial role in NAFLD progression (61). The higher amounts of IL-6 in MAT might directly or indirectly contribute to hepatic damage similar to insulin resistance (62) and to the severity of NAFLD (63); therefore, reducing IL-6 in MAT is a potential strategy for preventing NAFLD. Our results show that there was an elevation of IL-6 in the MAT of DKO-HRCD mice, which was suppressed by BCX. Moreover, in the present study, we observed high amounts of the anti-inflammatory cytokine IL-4 in the MAT of the DKO-HRCD group, which were significantly reduced by BCX feeding. Of note, IL-4 has been demonstrated to contribute to obesity in humans (64, 65), and plays a role in improvement of adipose tissue dysfunction by countering proinflammatory functions (66). Our results suggest that both proinflammatory and anti-inflammatory responses were elevated in the MAT of DKO-HRCD mice and were suppressed by BCX. It has been suggested that fatty acid β -oxidation reduces the expression of IL-6 in adipocytes (67). Similarly, our results suggest that BCX may induce the fatty acid

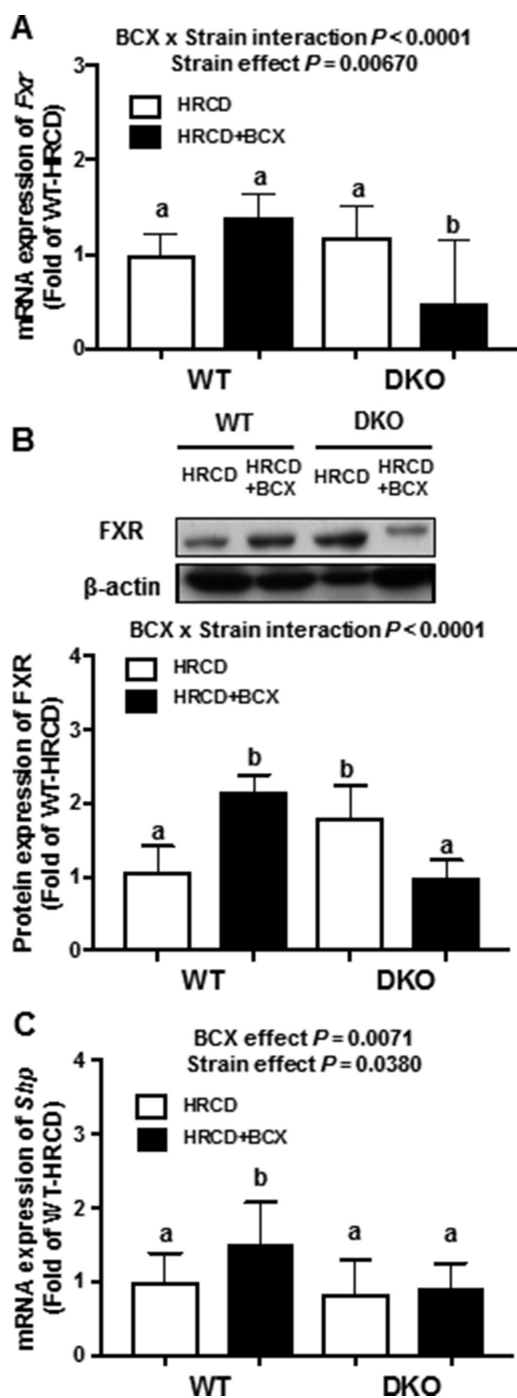


FIGURE 4 Effects of BCX on nuclear receptor FXR and SHP in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in the mRNA and protein expression of nuclear receptors. (A) *Fxr* mRNA ($n = 10-12$), (B) FXR protein ($n = 6$), and (C) *Shp* mRNA ($n = 10-12$) quantified via real-time PCR and Western blot in the livers of WT mice and DKO mice fed with HRCD supplemented with or without BCX. β -Actin was used as a loading control. Values are means $\pm$ SEMs, $n = 10-12$ except (B) where $n = 6$. Means without a common letter differ, $P < 0.05$. BCX, β -cryptoxanthin; DKO, double knockout; *Fxr*, farnesoid X receptor; HRCD, high-refined carbohydrate diet; *Shp*, small heterodimer partner; WT, wild-type.

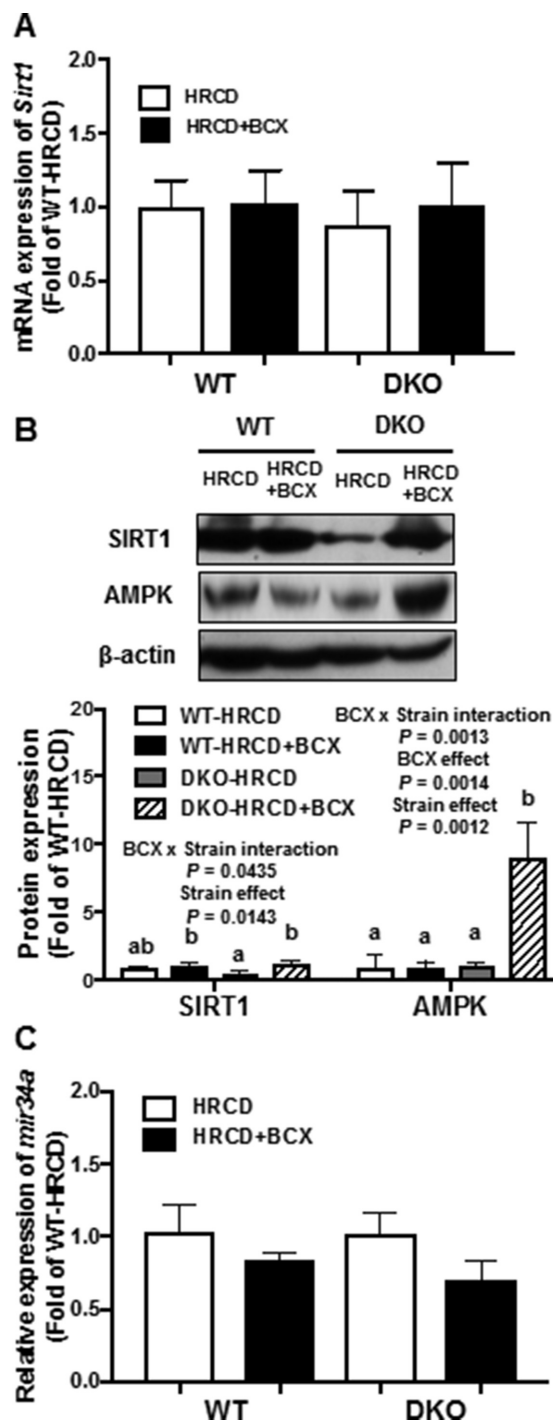


FIGURE 5 Effects of BCX on the SIRT1/AMPK pathway in WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in mRNA levels of (A) *Sirt1* and protein levels of (B) SIRT1, AMPK, and (C) *mir34a* quantified via real-time PCR and Western blot in the livers of WT mice and DKO mice fed with HRCD supplemented with or without BCX. β -Actin was used as a loading control in real-time PCR and Western blot testing. U6 snRNA was used as a loading control for *mir34a*. Values are means $\pm$ SEMs, $n = 10-12$ except (B) and (C) where $n = 6$. Means without a common letter differ, $P < 0.05$. BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; mir, microRNA; SIRT1, sirtuin 1; WT, wild-type.

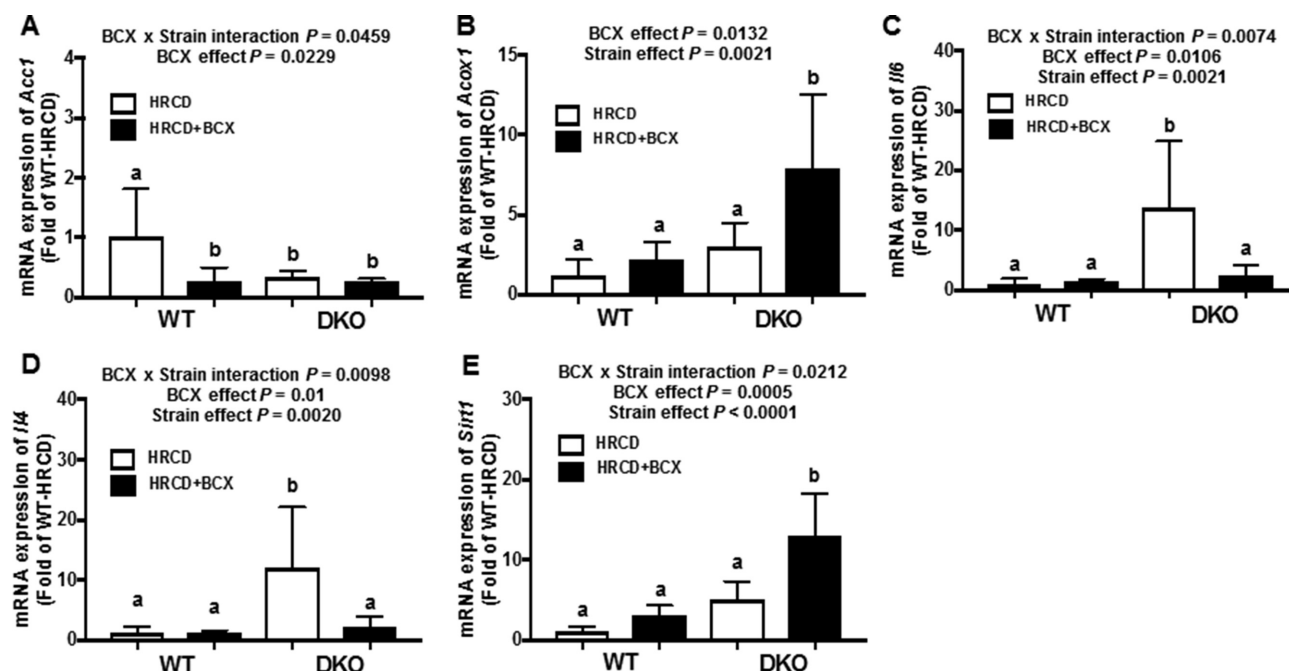


FIGURE 6 Effects of BCX on mesenteric lipid metabolism, inflammation, and SIRT1 in MAT of WT mice and BCO1/BCO2 DKO mice fed HRCD alone or supplemented with BCX. Graphical representation of fold changes in the mRNA expression of (A) *Acc1*, (B) *Acox1*, (C) *Il6*, (D) *Il4*, and (E) *Sirt1* quantified via real-time PCR in the livers of WT mice and DKO mice fed HRCD with or without BCX. β -Actin was used as loading control. Values are means $\pm$ SEMs, $n = 10$ – 12 . Means without a common letter differ, $P < 0.05$. *Acc1*, acyl CoA carboxylase1; *Acox1*, acyl CoA oxidase1; BCX, β -cryptoxanthin; DKO, double knockout; HRCD, high-refined carbohydrate diet; WT, wild-type.

β -oxidation marker (*Acox1*) in the MAT, which might lead to reduction of *Il6* in DKO mice (Figure 7B). The BCX-mitigated *Il6* in the MAT might be ascribed to upregulation of *Sirt1*, which is associated with the inhibition of inflammatory cytokines such as TNF- α and IL-6 in adipose tissues (33) and the regulation of peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α -regulated enzymes involved in fatty acid β -oxidation, including ACOX1 (68). Similarly, BCX administration has been reported to increase the fatty acid β -oxidation marker PPARA in visceral fat (23). In WT mice, we demonstrated that BCX significantly reduced the lipogenesis marker *Acc1* in the MAT (Figure 7A). Taken together, our findings imply that BCX differentially regulates lipid metabolism and pro- and anti-inflammatory cytokine amounts in the MAT, depending on the presence of BCO1/BCO2.

The dose of BCX (10 mg/kg diet) used in this study was equivalent to consumption of 0.87 mg/d [which would be easily achievable by consuming 85–113 g (or 1–2 medium-sized) of sweet red peppers] (40, 42). This dose is within the physiological range based on BCX in the WT-HRCD + BCX group, which had the carotenoid cleavage enzymes. The mean BCX in the livers of WT-HRCD + BCX mice (0.79 nmol/g tissue) was comparable to the BCX in human liver tissues (0.1–3.5 nmol/g tissue) (69). Our results are consistent with those reported from a clinical study in which the consumption of a beverage containing 3 mg/d of BCX by patients improved the metabolic risk parameters in NAFLD patients (20). We found that BCX in the DKO-HRCD + BCX mice was significantly higher than in the WT-HRCD + BCX mice. Considering that DKO mice do not have carotenoid cleavage enzymes, it was expected that DKO mice significantly accumulate BCX in the liver. Other studies have reported that

the accumulation of carotenoids in mice with either 1 of the carotenoid cleavage enzymes removed resulted in deleterious effects including oxidative stress (70) and fat accumulation (71). In contrast, we observed beneficial effects of BCX accumulation on HRCD-induced NAFLD in DKO mice; this could be because of the lower dose of BCX used in our study (10 mg/kg diet) compared to that used by others (~50 mg–1 g/kg diet) (70, 71).

Because BCX in DKO-HRCD + BCX mice was significantly (~33-fold) higher than in WT-HRCD + BCX mice, we cannot exclude the possibility that differences in the accumulation of BCX between the DKO and WT mice play a role in terms of the protective effect of BCX. Different amounts of BCX may also diversely impact specific molecular pathways. In addition, we did not detect any other carotenoids and BCX metabolites aside from vitamin A in both WT and DKO mice. Therefore, our data support that the inhibition of HRCD-induced NAFLD is mainly because of BCX.

In conclusion, the findings from this study suggest that BCX exerts protective effects against HRCD-induced NAFLD by regulating multiple molecular pathways in the liver-MAT axis. The molecular mechanisms differed in terms of BCX regulation, however, which depended on BCO1/BCO2, suggesting that intact BCX has a unique biological function in prevention of HRCD-induced NAFLD. Together, our results strongly suggest that the consumption of abundant fruits and vegetables enriched in BCX may be necessary to lower the risk of NAFLD associated with the high consumption of refined carbohydrate/sugar diets, regardless of the presence of BCO1/BCO2 polymorphisms in humans.

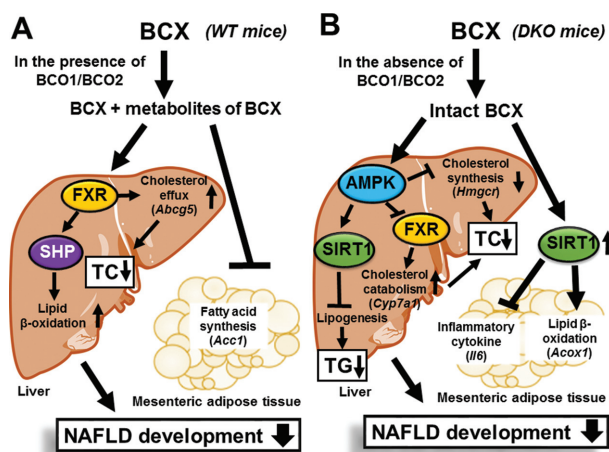


FIGURE 7 The proposed mechanism underlying the prevention of HRCD-mediated NAFLD by BCX in WT mice and BCO1/BCO2 DKO mice HRCD alone or supplemented with BCX. (A) In WT mice, BCX upregulates FXR and subsequently activates SHP, thus elevating fatty acid β -oxidation and cholesterol efflux in the liver. BCX also inhibits lipogenesis in the MAT. All these factors potentially contribute to the prevention of HRCD-induced NAFLD. (B) In DKO mice, BCX upregulates AMPK and subsequently activates SIRT1, thereby inhibiting lipogenesis and cholesterol biosynthesis in the liver. Intact BCX downregulates FXR, possibly by AMPK activation, thereby upregulating CYP7A1, potentially elevating cholesterol catabolism in the liver. BCX also upregulates SIRT1, thus inhibiting IL-6 and increasing fatty acid β -oxidation in the MAT. All these factors potentially contribute to the prevention of HRCD-induced NAFLD. BCX, β -cryptoxanthin; CYP7A1, cholesterol 7α -hydroxylase; DKO, double knockout; FXR, farnesoid X receptor; HRCD, high-refined carbohydrate diet; MAT, mesenteric adipose tissue; NAFLD, nonalcoholic fatty liver disease; SHP, small heterodimer partner; SIRT1, sirtuin1; WT, wild-type.

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