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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]. n 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/6 J genetic background revealed 83% C57BL/6 J genetic background. We then backcrossed the mice onto a C57BL/6 J background to generate $BCO1^{+/-}/BCO2^{+/-}$ heterozygous F1 generation mice (estimated to have a 93% C57BL/6 J 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].