Cardiac dysfunction in \( \beta \)-carotene-15,15'-dioxygenase-deficient mice is associated with altered retinoid and lipid metabolism

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Lee S, Jiang H, Trent CM, Yuen JJ, Narayanasamy S, Curley RW, Jr., Harrison EH, Goldberg JJ, Maurer MS, Blaner WS. Cardiac dysfunction in \( \beta \)-carotene-15,15'-dioxygenase-deficient mice is associated with altered retinoid and lipid metabolism. Am J Physiol Heart Circ Physiol 307: H1675–H1684, 2014. First published September 26, 2014; doi:10.1152/ajpheart.00548.2014.—Dietary carotenoids that are collectively known as provitamin A carotenoids, such as \( \beta \)-carotene (7, 16, 29). Most tissues maintain some retinoid stores, primarily as retinyl esters, but for healthy well-nourished organisms, the liver accounts for 80–90% of all retinoid present in the body (7, 29). Liver retinoid stores are used physiologically to maintain blood retinoid levels constant (7, 29). This involves the secretion of retinol to the specific circulating binding protein, retinol-binding protein 4 (RBPA), which delivers retinol to tissues, where it is taken up and either stored or enzymatically oxidized to ATRA (34, 38). Provitamin A carotenoids like \( \beta \)-carotene are taken up by the intestine and cleaved in situ by the enzyme \( \beta \)-carotene-15,15'-dioxygenase (BCO1) to retinoid, which is then metabolically indistinguishable from dietary preformed vitamin A (6, 11, 43). In humans, some dietary provitamin A carotenoids is absorbed intact into the body, along with other dietary lipids, in nascent chylomicrons and distributed to tissues throughout the body (6, 11, 43). There are no specific tissue sites for carotenoid storage per se, but some tissues, like adipose tissue, accumulate higher concentrations of carotenoids than others. Moreover, many tissues express Bco1 mRNA and protein, and this has been proposed to allow for the cleavage of provitamin A carotenoids to retinoids within these tissues (31, 35, 44, 48, 49). In addition to BCO1, one other mammalian enzyme is known to be able to metabolize carotenoids: \( \beta \)-carotene-9',10'-dioxygenase 2 (BCO2) (15, 16, 21, 22). Unlike BCO1, which catalyzes \( \beta \)-carotene cleavage about its central 15–15' double bond, BCO2 catalyzes the asymmetric cleavage of \( \beta \)-carotene at the 9'-10' double bond, forming \( \beta \)-apo-10'-carotenal, one of the structurally distinct products that are collectively known as \( \beta \)-apo-carotenals (11, 18, 21, 44). These \( \beta \)-apo-carotenals can then either undergo enzymatic oxidation to corresponding \( \beta \)-apo-carotenonic acids or a reduction to corresponding \( \beta \)-apo- carotenols, which can subsequently undergo esterification or conversion to retinoid (4, 11, 18, 21, 44). Some \( \beta \)-apo-carotenoids are also formed nonenzymatically.

It has been known for several decades that the asymmetric \( \beta \)-apo-carotenoid cleavage products of \( \beta \)-carotene are present in human and animal blood and tissues (37, 45). To some degree, these compounds are destined for elimination from the body (3), but the extent of this has not been established. In 2007, Plutsky and colleagues (52, 53) reported that one of these compounds, \( \beta \)-apo-14'-carotenal, interacts with retinoid X receptors (RXRs) and both peroxisome proliferator-activated receptor (PPAR)-\( \alpha \) and PPAR-\( \gamma \) to oppose known effects of both synthetic and natural ligands of these receptors, thereby preventing target gene induction in vitro and functional cellular responses in vivo.

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that some apo-β-carotenoids, specifically β-apo-13-carotenone, β-apo-14'-carotenal, and β-apo-14'-'carotenomic acid, are very potent RAR antagonists, with binding affinities in the low nanomolar range. These binding affinities are similar in magnitude to that of ATRA for RARs (13). In addition, β-apo-13-carotenone is a potent antagonist of RXRs transactivation (12).

To gain better understanding of the metabolism and actions of β-apo-carotenoids within the body, we established liquid chromatography tandem mass spectrometry (LC/MS/MS) protocols for measuring β-apo-carotenoid levels in the mouse plasma, liver, and heart. We were interested in understanding if the lack of Bco1 expression might influence tissue β-apo-carotenoid levels. To this end, we used matched male wild-type (WT) and Bco1-deficient (Bco1−/−) mice in our investigations and studied one tissue that normally expresses Bco1, the liver, and one tissue that does not, the heart. Adult Bco1−/− mice reproduce normally but have been reported to develop hepatic steatosis more readily than WT mice (17), and female Bco1−/− mice fed a control diet display increased inflammation in their lungs compared with WT mice (42). By studying mice with a genetic ablation of Bco1, we discovered that the absence of Bco1 affects cardiac retinoid and lipid homeostasis and heart function.

MATERIALS AND METHODS

Animals, husbandry, and experimental diets. Experiments involving animals were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (27) and were approved by the Institutional Animal Care and Use Committee of Columbia University. For all of our experiments, we used male WT C57BL/6j mice (Jackson Laboratory) and Bco1−/− mice congenic in this inbred background. Genotypes of Bco1−/− mice, which were originally described for the mixed C57BL/6J;129svJ genetic background (14), have undergone 10 backcrosses with C57BL/6j mice to render them congenic in this inbred background. Genotypes of Bco1−/− mice were determined by PCR using a previously described protocol (14). Mice were housed in a pathogen-free animal facility under 12:12-h light-dark cycles at constant temperature and humidity and had ad libitum access to chow diet (5053 PicoLab Rodent Diet 20, Purina Nutritional Products) dispersed in PBS at a final concentration of 1 l of 1:1 (vol/vol) methyl t-butyl ether-methanol before LC/MS/MS.

Animals, husbandry, and experimental diets. Experiments involving animals were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (27) and were approved by the Institutional Animal Care and Use Committee of Columbia University. For all of our experiments, we used male WT C57BL/6j mice (Jackson Laboratory) and Bco1−/− mice congenic in this inbred background. Genotypes of Bco1−/− mice, which were originally described for the mixed C57BL/6J;129svJ genetic background (14), have undergone 10 backcrosses with C57BL/6j mice to render them congenic in this inbred background. Genotypes of Bco1−/− mice were determined by PCR using a previously described protocol (14). Mice were housed in a pathogen-free animal facility under 12:12-h light-dark cycles at constant temperature and humidity and had ad libitum access to chow diet (5053 PicoLab Rodent Diet 20, Purina Mills) containing 15 IU vitamin A/g diet. For β-carotene supplementation, we used water-soluble β-carotene beadlets (10% CWs, DSM Nutritional Products) dispersed in PBS at a final concentration of 1 mg/100 g. β-Carotene was administered to mice by intraperitoneal injection in a single dose given 3 days before euthanization. All manipulations involving β-carotene were carried out under red light.

LC/MS/MS analyses of β-apo-carotenoids, retinoids, and lipids. For analysis of β-apo-carotenoids and β-carotene levels in tissues, livers and hearts were homogenized in 4 volumes of PBS to render them 20% (wt/vol) homogenates. Internal standards (retinyl acetate and 11-C18-β-carotene) were then added in 1 ml of ethanol containing 0.1% (wt/vol) butylated hydroxytoluene as an antioxidant. Subsequently, 6 volumes of 10:6:7:7 hexane-ethanol-acetone-toluene (HEAT) were added along with saturated NaCl solution (200 μl) to facilitate phase separation. Samples were vortexed for 60 s and centrifuged at 5,000 g for 5 min at 4°C. The upper organic layer was removed, and the aqueous phase was extracted twice more by adding HEAT-NaCl. The three organic phases were combined and dried under a stream of nitrogen. The residue was reconstituted in 40 μl of 1:1 (vol/vol) methyl t-butyl ether-methanol before LC/MS/MS. An aliquot of whole plasma was extracted using the same protocol but scaled for the plasma volume.

LC/MS/MS analyses of plasma and tissue concentrations of β-apo-carotenoids and retinoid acid were conducted using a Waters Xevo TQ MS ACQUITY UPLC system controlled by MassLynx software (version 4.1). Samples were maintained at 4°C in the autosampler, and 5 μl were loaded onto a Waters ACQUITY UPLC BEH Phenyl column (3.0 × 100 mm, 1.7-μm particle size) for analysis of β-apo-carotenals or β-apo-carotenolic acids or an ACQUITY UPLC BEH HSS column (3.0 × 100 mm, 1.7-μm particle size) for analysis of β-apo-13-carotenone and ATRA. The running columns were preceded by a 2.1 × 5-mm guard column containing the same packing material. Columns were maintained at 40°C. Separations were achieved using water/acetonitrile gradients in the presence of formic acid for β-apo-13-carotenone and ATRA or water/methanol gradients in the presence of formic acid and ammonium formate for β-apo-carotenals or β-apo-carotenolic acids. Positive ESI-MS in the multiple reaction monitoring mode was performed. Concentrations of β-apo-carotenoids in serum and tissues were determined by comparing integrated peak areas for each compound against those of known amounts of purified standards that were chemically synthesized as previously described (11, 13). Loss of β-apo-carotenoids during extraction was accounted for by adjusting for the recovery of the internal standard retinyl acetate added before solvent extraction.

Retoic acid concentration in livers of mice were determined as we have previously reported (47). Penta-deuterated ATRA (ATRA-d5) was used as an internal standard and was purchased from Toronto Research Chemicals (North York, ON, Canada).

Free fatty acid (FFA), ceramide, endocannabinoid, and N-acetylenalalalaminolamine (NAE) concentrations were determined by LC/MS/MS according to protocols we have previously described in detail (9). mRNA isolation and quantitative RT-PCR analysis. Total RNA was isolated from the heart and liver using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples were DNase I digested using the RNeasy mini kit (Qiagen) to remove residual genomic DNA and subsequently quantified using a NanoDrop spectrophotometer. For cDNA synthesis, 1 μg of total RNA was reverse transcribed using the High Capacity Reverse Transcription Kit (Applied Biosystems). cDNA synthesis was carried out for 10 min at 20°C and 120 min at 37°C. The reaction was stopped by incubation at 85°C for 5 min using a thermal cycler (Eppendorf). Sequences for the primers used in this study have been previously published (9). Quantitative RT-PCR was performed in a total volume of 25 μl containing diluted cDNA template (1:40), forward and reverse primers (1 μM each), and LightCycler 480 SYBR Green I Master Mix (Roche). Expression levels of target genes are presented relative to the reference gene 18S. A dissociation curve program was used after each reaction to verify the purity of the PCR products.

Immunoblot analysis. Dissected perfused livers and hearts were homogenized in RIPA buffer containing protease inhibitor cocktails (Sigma) with 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% (wt/vol) SDS, 0.5% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, and 1 mM EDTA. Cell lysates were obtained after centrifugation at 15,000 g for 15 min at 4°C. Total protein extract (30 μg) was applied for SDS-PAGE and transferred onto polyvinylidene difluoride membranes for subsequent probing with polyclonal antisera against mouse BCO1 (1:2,000 dilution) (31) in conjugation with horseradish peroxidase-conjugated secondary antibody and an ECL detection system (Thermo Scientific). Protein loading was normalized using horseradish peroxidase-conjugated monoclonal α-tubulin antibody (1:1,000, Santa Cruz Biotechnology).

Plasma and tissue triglyceride and total cholesterol measurements. Blood was collected from mice for measurements of plasma triglycerides (TGs), total cholesterol (TC), and nonesterified fatty acids (FFAs). TGs and TC from plasma and pieces of the liver and myocardium were measured enzymatically using Infinity kits (ThermoFisher Scientific) according to the manufacturer’s instructions. FFAs were measured using a NEFA kit (Wako Pure Chemical Industries) according to the manufacturer’s instructions. For measurements of tissue lipids, livers and hearts were first perfused with PBS...
and then homogenized at 4°C in PBS. Lipids were extracted from ~50 mg tissue in 6 volumes of 2:1 (vol/vol) chloroform-methanol. Chloroform-extractable lipids were dried under a gentle stream of N2, and subsequently solubilized in water containing 2% (vol/vol) Triton X-100, and aliquots taken for assay.

**Echocardiography.** Two-dimensional echocardiography was performed using a high-resolution imaging system with a 30-MHz imaging transducer (Vevo 770, VisualSonics) in unconscious 19-wk-old male wild-type (WT) mice. Mice were anesthetized with 1.5–2% isoflurane and thereafter maintained on 1–1.5% isoflurane throughout the procedure. Two-dimensional echocardiographic images were obtained and recorded in a digital format. Images were then analyzed offline by a single observer blinded to the murine genotype. Left ventricular (LV) end-diastolic dimension (LVDd) and LV end-systolic dimension (LVDs) were measured. The ejection fraction (EF) was calculated as follows: EF (in %) = [(LVDd - LVDs)/LVDd] × 100%. Fractional shortening (FS), which quantifies contraction of the ventricular wall and is an indication of muscle function, was calculated as follows: FS (%) = [(LVDd - LVDs)/LVDd] × 100%.

**Statistical analysis.** Data are presented as means ± SE. Student’s t-test was used to identify differences between control and knockout strains. P values of <0.05 were considered statistically significant.

**RESULTS**

To understand how Bco1 expression affects concentrations of β-apo-carotenals and β-apo-carotenoic acids within tissues and blood, we set up LC/MS/MS methods for assessing β-apo-carotenoid levels. We developed a method that allowed us to chromatographically separate and detect chemically synthesized standards for β-apo-14′-carotenals and β-apo-14′-carotenoids. We used these standards to detect and quantify these β-carotene metabolites in extracts prepared from the plasma, liver, and heart of chow-fed adult mice. We did not observe matrix effects for these tissues, as assessed by determinations of recoveries and separations for liver, heart, and plasma extracts spiked with different concentrations of authentic β-apo-carotenoid standards.

We focused on the liver and heart since the adult liver but not the adult heart expresses BCO1 protein, whereas the adult heart does not (Fig. 1). The liver and heart have both been reported to express BCO2 (18). All 10 β-apo-carotenoids were present in the liver, heart, and plasma of 4- to 5-mo-old male chow-fed WT and Bco1−/− mice. To facilitate understanding of these data, we summed individual tissue levels (as shown in Fig. 2) as total β-apo-carotenal and total β-apo-carotenoic acid levels. As shown in Fig. 2, A and B, levels of both total β-apo-carotenals and β-apo-carotenoic acids were significantly lower in livers of Bco1−/− compared with WT mice, whereas, no statistically significant differences in these levels were observed for the heart (Fig. 2, C and D). Plasma β-apo-carotenals were significantly lower in Bco1−/− mice compared with WT (Fig. 2E), but no effect of genotype on plasma β-apo-carotenoic acid levels was observed (Fig. 2F). No statistically significant differences in liver, heart, or plasma levels of β-apo-13-carotenone were detected for Bco1−/− and WT mice fed a chow diet (data not shown). Tissue levels of individual β-apo-carotenals and β-apo-carotenoic acids are shown in Tables 1–3.

Hessel et al. (17) reported an elevation of hepatic total lipids and TGs in Bco1−/− mice compared with WT mice. To understand whether there are relationships between plasma or tissue β-apo-carotenoid concentrations and hepatic lipids, we measured TGs, FFAs, TC, and free cholesterol levels in livers, hearts, and plasma of our chow-fed experimental mice. In contrast to Hessel et al. (17), we did not observe differences in hepatic TG or FFA levels (Fig. 3, A and B) or hepatic TC levels (data not shown) between matched Bco1−/− and WT mice. Nor did we observe differences between knockout and WT mice in plasma levels of these lipids (data not shown). Surprisingly, though, heart TG levels were significantly lower in Bco1−/− mice than in matched WT mice (Fig. 3C). This was accompanied by a significant elevation in heart levels of FFAs (Fig. 3D). We further analyzed, by LC/MS/MS, the acyl composition of the FFA pool within the heart. Many individual FFA species were modestly but significantly elevated in Bco1−/− hearts, including C16:0, C16:1, C18:1, C18:2, and C22:6 (Fig. 3E).

These lipid changes could suggest that intracardiac TG lipolysis is increased in the Bco1−/− heart. However, we did not detect any differences in intracellular lipase expression for either hormone-sensitive lipase or adipocyte triglyceride lipase (Fig. 3F). Surprisingly, mRNA levels for lipoprotein lipase (Lpl) and the FFA uptake transporter Cd36 were increased (Fig. 3F), suggesting that there is increased FFA uptake from plasma of the Bco1−/− heart. We also observed increased expression of genes important for FFA synthesis and metabolism in the Bco1−/− heart, including Pparγ (Fig. 3F) as well as stearoyl-CoA desaturase 1 (Scd1) and fatty acid synthase (Fas) (Fig. 3G). Interestingly, expression of diacylglycerol O-acyltransferase 2 (Dgat2), which encodes an enzyme important for TG synthesis, was also found to be elevated in Bco1−/− hearts, although TG levels were significantly lower in these hearts.

Increased heart FFAs with decreased TG storage might have led to the incorporation of FFAs into other lipids. For this reason, we assessed levels of ceramides and found that these too were elevated in hearts of Bco1−/− mice compared with WT mice (Fig. 3H). We also identified a statistically significant increase in total NAE species (the sum of arachidonoyl, myristoyl, palmitoyl, lineoloyl, oleoyl, and stearoyl

**Fig. 1.** β-Carotene-15,15′-dioxygenase (BCO1) protein is not expressed in adult hearts of male wild-type (WT) mice. Western blot analysis of BCO1 and α-tubulin protein levels in extracts prepared from hearts and livers of matched 19-wk-old WT and Bco1-deficient (Bco1−/−) mice is shown. As a positive control (PC), we used homogenates prepared from Chinese hamster ovary cells overexpressing mouse BCO1.

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**Table 1.** Carotenoid levels in liver and heart of chow-fed experimental mice. (Data not shown)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Bco1−/−</th>
<th>WT</th>
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<tbody>
<tr>
<td>Liver</td>
<td>10−10</td>
<td>10−10</td>
</tr>
<tr>
<td>Heart</td>
<td>10−10</td>
<td>10−10</td>
</tr>
</tbody>
</table>

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ethanolamide levels measured individually) in hearts of Bco1−/− mice (Fig. 3I). However, levels of the two canonical endocannabinoids, arachidonoyl ethanolamide and 2-arachidonoyl glycerol, were not significantly different in mutant hearts (data not shown).

Since we observed marked differences in Bco1−/− heart lipid levels, including potentially toxic ceramides, we carried out an experiment to determine whether heart functions may be compromised in Bco1−/− mice. We identified differences in a number of parameters associated with heart function by two-dimensional echocardiography analysis of Bco1−/− and WT mice (Fig. 4, A–E). Hearts from Bco1−/− mice had decreased FS, decreased EF, and increased LVDd. Whereas LVDd was not affected (WT vs. Bco1−/− mice: 3.19 ± 0.34 vs. 3.54 ± 0.41, P = not significant), LVDs was significantly increased (WT vs. Bco1−/− mice: 1.31 ± 0.35 vs. 2.15 ± 0.72, P < 0.05), suggesting that Bco1−/− mice have reduced cardiac contractility. FS [FS (%)] = [(LVDd – LVDs)/LVDd] × 100% observed for Bco1−/− mice clearly indicated that systolic dysfunction (decreased FS due to increased systolic diameter) was the primary cause of the impaired cardiac contractility.

We also investigated possible gene expression changes that might be associated with heart functions. We observed a significant increase in mRNA expression levels for brain natriuretic peptide (BNP) in Bco1−/− mice (Fig. 4F). No differ-

Table 1. β-Apo-carotenoid levels in livers of WT mice versus Bco1−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>BC (1 mg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT mice, pmol/g tissue</td>
<td>Bco1−/− mice, pmol/g tissue</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>β-13-Carotene</td>
<td>11.4 ± 7.2</td>
<td>4.6 ± 4.0</td>
<td>0.12</td>
</tr>
<tr>
<td>β-Apo-14-carotenal</td>
<td>2,870 ± 390</td>
<td>1,178 ± 137</td>
<td>0.0002***</td>
</tr>
<tr>
<td>β-Apo-14-carotenoid acid</td>
<td>504 ± 87</td>
<td>392 ± 115</td>
<td>0.17</td>
</tr>
<tr>
<td>β-Apo-12-carotenol</td>
<td>16.3 ± 2.4</td>
<td>17.1 ± 3.0</td>
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<tr>
<td>β-Apo-12-carotenoid acid</td>
<td>253 ± 29</td>
<td>179 ± 19</td>
<td>0.002**</td>
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<tr>
<td>β-Apo-10-carotenol</td>
<td>15.6 ± 4.0</td>
<td>19.4 ± 5.9</td>
<td>0.31</td>
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<td>β-Apo-10-carotenoid acid</td>
<td>22.2 ± 5.9</td>
<td>28.9 ± 5.7</td>
<td>0.13</td>
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<tr>
<td>β-Apo-8′-carotenol</td>
<td>57.7 ± 8.5</td>
<td>49.6 ± 6.5</td>
<td>0.15</td>
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<tr>
<td>β-Apo-8′-carotenoid acid</td>
<td>56.3 ± 12</td>
<td>41.9 ± 13.7</td>
<td>0.17</td>
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</tbody>
</table>

Concentrations of compounds are provided as means ± SD. WT mice, wild-type mice; Bco1−/− mice, β-carotene-15,15′-dioxigenase-deficient mice; BC, β-carotene. Significantly different from the corresponding concentration in WT mice: **P < 0.01, and ***P < 0.005.
ences in heart mRNA expression levels for glucose transporter 4, pyruvate dehydrogenase kinase 4, α-myosin heavy chain, or β-myosin heavy chain were observed. Histological analysis of heart slices did not show differences between Bco1−/− and WT mice with regards to overall structure (data not shown).

Heart levels of retinol but not retinyl esters were significantly elevated in Bco1−/− mice (Fig. 5, A and B). This was accompanied by increased mRNA expression of Rbp4 but not Rbp1, Rara, Rarb, Rarg, or lecithin retinol acyltransferase (Fig. 5C). Interestingly, heart expression of retinol dehydrogenase 10, a gene that encodes the enzyme responsible for catalyzing the first oxidative step needed for forming ATRA from all-trans-retinol, was significantly elevated. Expression of other genes involved in ATRA synthesis and catabolism [the cytochrome P-450 (Cyp) family members Cyp26a1 and Cyp26b1, aldehyde dehydrogenase 1 family member A2, or dehydrogenase/reductase (SDR family) member 3] were not different (Fig. 5D). Heart β-carotene levels were also elevated in Bco1−/− mice (Fig. 5E), as were mRNA levels for Bco2 (Fig. 5F). Bco2 mRNA levels were not different in the liver (data not shown). Although heart ATRA levels tended to be lower for chow-fed Bco1−/− mice than in WT mice, this did not reach statistical significance (Fig. 5G).

We then tested whether a large dose of water-soluble β-carotene (1 mg), given 3 days before euthanization of the mice, might affect plasma and tissue β-apo-carotenoids. As shown in Fig. 2, β-carotene supplementation resulted in elevated liver total β-apo-carotenoid and total β-apo-carotenoid acid concentrations and in elevated heart and plasma β-apo-carotenal levels for both Bco1−/− and WT mice. β-Carotene supplementation did not markedly affect the relative rank orders of the concentrations of individual β-apo-carotenoids in the three tissues (Tables 1–3). For mice that received this dose (1 mg) of β-carotene 3 days before euthanization, we also observed statistically significant decreases in heart ATRA concentrations for both Bco1−/− and WT mice (Fig. 5G).

### DISCUSSION

Eroglu et al. (11, 13) established that some eccentric cleavage products of β-carotene are potent antagonists of ATRA and RAR transcriptional regulation. This finding raises the possibility that some eccentric cleavage products of β-carotene may act physiologically to modulate retinoid transcriptional activity. To better understand the physiology of β-carotene cleavage products, we established very sensitive LC/MS/MS methods that allowed us to assess tissue levels of 10 eccentric β-carotene cleavage products, including the potent antagonists β-apo-14′-carotenal and β-apo-13-carotene. To our knowledge, no systematic studies of tissue levels of β-apo-carotenal or β-apo-carotenoid acid levels have been reported in the literature. Thus, we determined the levels of these compounds in the liver, heart, and plasma of 4- to 5-mo-old chow-fed male mice. We note that a rodent chow diet contains carotenoids that are added as plant materials to the diet, for instance, dehydrated alfalfa meal in the case of the chow diet we used in our study. As shown in Fig. 2 and Tables 1–3, compared with the heart, the liver possesses higher concentrations of a majority of the individual β-apo-carotenoids as well as total β-apo-carotenals and total β-apo-carotenoid acids. Plasma levels for each of the β-apo-carotenoids were either lower or at the same level as

### Table 2. β-Apo-carotenoid levels in hearts of WT mice versus Bco1−/− mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chow</th>
<th>Bco1−/−</th>
<th>P value</th>
<th>BC (1 mg)</th>
<th>Bco1−/−</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT mice, pmol/g tissue</td>
<td>Bco1−/− mice, pmol/g tissue</td>
<td>P value</td>
<td>WT mice, pmol/g tissue</td>
<td>Bco1−/− mice, pmol/g tissue</td>
<td>P value</td>
</tr>
<tr>
<td>β-13-Carotene</td>
<td>1.9 ± 0.3</td>
<td>2.0 ± 0.3</td>
<td>0.82</td>
<td>2 ± 0.9</td>
<td>2.3 ± 0.6</td>
<td>0.91</td>
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<tr>
<td>β-Apo-14′-carotenal</td>
<td>257 ± 43</td>
<td>289 ± 75</td>
<td>0.49</td>
<td>494 ± 114</td>
<td>349 ± 120</td>
<td>0.11</td>
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<tr>
<td>β-Apo-14′-carotenoid acid</td>
<td>463 ± 127</td>
<td>574 ± 82</td>
<td>0.16</td>
<td>564 ± 159</td>
<td>489 ± 70</td>
<td>0.38</td>
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<tr>
<td>β-Apo-12′-carotenal</td>
<td>12 ± 8.8</td>
<td>12 ± 4.1</td>
<td>0.94</td>
<td>58 ± 10</td>
<td>103 ± 23</td>
<td>0.02*</td>
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<tr>
<td>β-Apo-12′-carotenoid acid</td>
<td>404 ± 61</td>
<td>405 ± 62</td>
<td>0.99</td>
<td>390 ± 74</td>
<td>358 ± 76</td>
<td>0.55</td>
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<tr>
<td>β-Apo-10′-carotenal</td>
<td>17 ± 3.5</td>
<td>19 ± 8.0</td>
<td>0.61</td>
<td>33 ± 8.0</td>
<td>110 ± 28</td>
<td>0.009**</td>
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<td>β-Apo-10′-carotenoid acid</td>
<td>18 ± 50</td>
<td>30 ± 35</td>
<td>0.04*</td>
<td>56 ± 16</td>
<td>85 ± 8.9</td>
<td>0.01*</td>
</tr>
<tr>
<td>β-Apo-8′-carotenal</td>
<td>3 ± 0.8</td>
<td>4.3 ± 1.2</td>
<td>0.20</td>
<td>10 ± 1.6</td>
<td>8.2 ± 1.6</td>
<td>0.17</td>
</tr>
<tr>
<td>β-Apo-8′-carotenoid acid</td>
<td>10 ± 4.1</td>
<td>21 ± 6.2</td>
<td>0.03*</td>
<td>52 ± 13</td>
<td>41 ± 7.6</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Concentrations of compounds are provided as means ± SD. Significantly different from the corresponding concentration in WT mice: *P < 0.05, **P < 0.01, and ***P < 0.005.

### Table 3. β-Apo-carotenoid levels in plasma of WT mice versus Bco1−/− mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chow</th>
<th>Bco1−/−</th>
<th>P value</th>
<th>BC (1 mg)</th>
<th>Bco1−/−</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT mice, nM</td>
<td>Bco1−/− mice, nM</td>
<td>P value</td>
<td>WT mice, nM</td>
<td>Bco1−/− mice, nM</td>
<td>P value</td>
</tr>
<tr>
<td>β-13-Carotene</td>
<td>0.4 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.01*</td>
<td>0.6 ± 0.3</td>
<td>1.4 ± 0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>β-Apo-14′-carotenal</td>
<td>101 ± 17</td>
<td>69 ± 15</td>
<td>0.02*</td>
<td>217 ± 29</td>
<td>263 ± 57</td>
<td>0.22</td>
</tr>
<tr>
<td>β-Apo-14′-carotenoid acid</td>
<td>472 ± 24</td>
<td>455 ± 34</td>
<td>0.45</td>
<td>655 ± 186</td>
<td>991 ± 359</td>
<td>0.14</td>
</tr>
<tr>
<td>β-Apo-12′-carotenal</td>
<td>2.9 ± 0.6</td>
<td>3.2 ± 0.4</td>
<td>0.40</td>
<td>15 ± 4.6</td>
<td>31 ± 5.1</td>
<td>0.003***</td>
</tr>
<tr>
<td>β-Apo-12′-carotenoid acid</td>
<td>131 ± 29</td>
<td>119 ± 18</td>
<td>0.46</td>
<td>43 ± 9.1</td>
<td>44 ± 12</td>
<td>0.95</td>
</tr>
<tr>
<td>β-Apo-10′-carotenol</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.45</td>
<td>0.3 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.001***</td>
</tr>
<tr>
<td>β-Apo-10′-carotenoid acid</td>
<td>34 ± 5.0</td>
<td>51 ± 7.2</td>
<td>0.008**</td>
<td>50 ± 6.7</td>
<td>73 ± 13</td>
<td>0.03*</td>
</tr>
<tr>
<td>β-Apo-8′-carotenol</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>0.21</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.46</td>
</tr>
<tr>
<td>β-Apo-8′-carotenoid acid</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.89</td>
<td>4.4 ± 0.6</td>
<td>3.6 ± 0.9</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Concentrations of compounds are provided as means ± SD. Significantly different from the corresponding concentration in WT mice: *P < 0.05, **P < 0.01, and ***P < 0.005.
those found in the liver and/or heart. The distributions and levels of these metabolites in the liver, heart, and plasma appear to be both tissue and metabolite dependent. However, metabolite levels do not segregate into distinct biochemical (structural) patterns that readily lend themselves to inferences.

Administration of a 1-mg bolus dose of \( ^{14} \)H\textsubscript{9252}-carotene (per body weight, equivalent to administering 2.8 g \( ^{14} \)H\textsubscript{9252}-carotene to a 70-kg human) 3 days before euthanization resulted in a very marked elevation in hepatic total \( ^{14} \)H\textsubscript{9252}-apo-carotenal levels and increased hepatic total \( ^{14} \)H\textsubscript{9252}-apo-carotenoic acid levels. Although this doubled heart total \( ^{14} \)H\textsubscript{9252}-apo-carotenal levels, there was no affect on heart total \( ^{14} \)H\textsubscript{9252}-apo-carotenoic acid concentrations. Plasma total \( ^{14} \)H\textsubscript{9252}-apo-carotenal levels were also doubled in \( ^{14} \)H\textsubscript{9252}-carotene-supplemented mice compared with unsupplemented mice. These data establish that tissue \( ^{14} \)H\textsubscript{9252}-apo-carotenoid levels are responsive to \( ^{14} \)H\textsubscript{9252}-carotene supplementation. Surprisingly, though, unlike mice fed a chow diet, we observed no differences for the three tissues examined in total \( ^{14} \)H\textsubscript{9252}-apo-carotenoid levels for \( Bco1^{−/−} \) versus WT mice. This could be explained if the metabolic capacity of the liver, heart, and other tissues to catabolize/eliminate these \( \beta \)-carotene metabolites is saturated owing to the large amount of \( \beta \)-carotene administered to the animals.

Most impressively, we found that \( Bco1^{−/−} \) mice develop a cardiac phenotype. We unexpectedly observed significantly diminished TG levels in hearts of \( Bco1^{−/−} \) mice, and this was accompanied by an elevation in heart FFA, ceramide, and NAE levels. Analysis of the acyl composition of the FFAs in \( Bco1^{−/−} \) hearts showed that most FFA species were elevated, suggesting that the elevation in FFAs was not due to a block in a specific arm of fatty acid metabolism, i.e., de novo lipogenesis or fatty acid elongation. We observed elevations in heart mRNA levels for both Lpl and Cd36. We take this finding to indicate that \( Bco1^{−/−} \) hearts are taking up more FFAs from the circulation. We also observed elevated expression of Pparg, Fas, and Scd1, genes involved in FFA synthesis and desaturation. Collectively, these findings suggest that elevated FFA levels in \( Bco1^{−/−} \) hearts arise through both increased endogenous FFA synthesis and increased FFA uptake from the circulation. Paradoxically, given the significantly lower TG

Fig. 3. Liver and heart levels of lipids for age- and genetic background-matched chow-fed male WT and \( Bco1^{−/−} \) mice. Liver triglyceride (TG; A) and total nonesterified fatty acids [free fatty acid (FFA); B] concentrations were not different for WT and \( Bco1^{−/−} \) mice, whereas heart TG (C) concentrations were diminished and total FFAs (D) were elevated in \( Bco1^{−/−} \) mice compared with WT mice. A number of relatively abundant individual FFA species were also significantly elevated in \( Bco1^{−/−} \) hearts (E). Expression of peroxisome proliferator-activated receptor (PPAR)-\( \gamma \) (Pparg), lipoprotein lipase (Lpl), comparative gene identification 58 (Cgi-58), and Cd36 mRNAs but not PPAR-\( \alpha \) (Ppara), hormone-sensitive lipase (Hsl), or adipocyte triglyceride lipase (Atgl) were significantly elevated in \( Bco1^{−/−} \) hearts (F). Elevated expression for genes involved in FFA synthesis and metabolism, including stearoyl-CoA desaturase 1 (Scd1), fatty acid synthase (Fas), and diacylglycerol O-acyltransferase 2 (Dgat2), were also elevated in \( Bco1^{−/−} \) hearts (G). Total ceramide (H) and total N-acylethanolamine (NAE; I) concentrations were also significantly elevated in hearts of \( Bco1^{−/−} \) mice. Values are means ± SE; \( n = 5–8 \). * \( P < 0.05 \); ** \( P < 0.01 \).
levels in Bco1<sup>−/−</sup> hearts, we observed elevated expression of Dgat2, one of two enzymes that catalyze the final step of TG synthesis. This is possibly a futile attempt aimed at allowing greater TG accumulation in the heart. Our observations are consistent with findings from Dixon et al. (10), who reported similar differences in lipids present in whole day 14.5 postcoitum Bco1<sup>−/−</sup> embryos. These authors found lower levels of TGs, cholesteryl esters, and phospholipid species in Bco1<sup>−/−</sup> embryos and suggested that BCO1 may have a direct role in regulating esterification (formation) of these lipid species (10).

Since FFAs comprise the major fuel for the adult heart and ceramides are known to be potentially toxic to the heart (inducing apoptosis) (33), we wondered whether differences in heart TG, FFA, and ceramide levels might be associated with

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**Fig. 4.** Heart functions are impaired in Bco1<sup>−/−</sup> mice as determined by two-dimensional echocardiography. A–D: percent fractional shortening (A), percent ejection fraction (B), left ventricular (LV) systolic diameter (LVDs; C), and LV diastolic diameter (LVDd; D) as measured by two-dimensional echocardiography for 19-wk-old chow-fed male WT and Bco1<sup>−/−</sup> mice in the C57BL/6 genetic background. E: photographs of representative echocardiograms for matched WT and Bco1<sup>−/−</sup> mice. F: heart brain natriuretic peptide (BNP) mRNA levels for WT and Bco1<sup>−/−</sup> mice as determined by quantitative RT-PCR analysis. AU, arbitrary units. Values are means ± SE; n = 5–8. *P < 0.05; **P < 0.01.

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**Fig. 5.** Heart retinoid and β-carotene levels as well as expression levels for genes associated with retinoid physiology. Heart levels of retinol (ROL; A) and β-carotene (E) were significantly elevated in Bco1<sup>−/−</sup> mice compared with WT mice. Although heart retinyl ester (RE) levels tended to be higher for Bco1<sup>−/−</sup> mice, this did not reach statistical significance (B). Of the genes encoding enzymes and binding proteins involved in retinoid metabolism, only retinol-binding protein 4 (Rbp4; C) and retinol dehydrogenase 10 (Rdh10; D) were expressed at significantly different levels in hearts of WT and Bco1<sup>−/−</sup> mice. Heart levels of all-trans-retinoic acid (RA) were not significantly different for Bco1<sup>−/−</sup> versus WT mice fed a chow diet (G). For both Bco1<sup>−/−</sup> and WT mice, heart all-trans-retinoic acid levels were significantly diminished upon administration of a single dose of β-carotene (1 mg) given 3 days before euthanization. Gene expression data established that only levels for Rbp4 mRNA (E) and β-carotene-9',10'-oxygenase 2 (Bco2) mRNA (F) were significantly different for Bco1<sup>−/−</sup> mice versus matched WT mice. Rara, Rarb, and Rarg, retinoic acid receptor-α, -β, and -γ, respectively; Lrat, lecithin retinol acyltransferase; Cyp, cytochrome P-450; Ralhd2, aldehyde dehydrogenase 1 family member A2; Dhrs3, dehydrogenase/reductase (SDR family) member 3. Values are means ± SE; n = 5–8. *P < 0.05; **P < 0.01; ***P < 0.001.
other differences in heart physiology. To assess this possibility, we undertook high-resolution echocardiography experiments to assess heart functions for 4- to 5-mo-old chow-fed male Bco1−/− mice and WT mice. This analysis revealed altered cardiac parameters (decreased FS and increased LVDs) in hearts of Bco1−/− mice. These findings indicate that Bco1−/− mice have compromised heart function, characterized by reduced cardiac contractility.

What is the molecular basis for the altered cardiac contractility associated with Bco1 deficiency? We suggest there are at least three possible explanations, each invoking a retinoid-dependent mechanism. It is well established that ATRA and RAR signaling are required for ensuring normal heart development in utero and for maintaining a healthy heart after birth. Developmental cardiovascular defects have been reported for animal models that received either retinoid-deficient or retinoid-excess diets and in retinoid receptor knockout embryos (20, 25, 28, 32). These developmental defects include ventricular septal defects, atrial septal defects, abnormal aortic arch patterning, atrioventricular canal defects, and ventricular chamber hypoplasia (25, 28, 31, 32). Interestingly, Osuala et al. (30) reported that targeted disruption of the gene for dopamine β-hydroxylase results in embryonic lethality due to heart failure. This was associated with a marked downregulation of Bco1 mRNA expression in the embryonic heart and effects on ATRA levels. Possibly, the differences we observed for 4- to 5-mo-old mice reflect congenital effects arising from altered retinoid availability in utero. To verify this possibility will require a systematic study of heart development in Bco1−/− mice.

In the adult, ATRA-mediated signaling pathways have an important role in regulating cardiac remodeling, suppressing cardiac hypertrophic features, including increased total protein content, protein synthesis, cell size, and myofibrillar reorganization (32). ATRA signaling has been reported by Bilbija et al. (5) to be activated in the mouse heart after permanent coronary artery ligation. These investigators further proposed that ATRA signaling may play a role in regulating damage and repair during heart remodeling (5). Minicucci et al. (26) reported that local heart retinoid insufficiency is associated with intensified ventricular remodeling after experimental myocardial infarction, worsening diastolic dysfunction. These differences were accompanied by an increase in BNP expression. Our data indicate that BNP expression is elevated in the Bco1−/− heart (Fig. 4F). BNP has been proposed to be a cardioprotective hormone that prevents pathological hypertrophy, fibrosis, and apoptosis of cardiomyocytes, and its expression has recently been shown to be induced by retinoids (23, 40). Thus, it is possible that an alteration in retinoid homeostasis in the Bco1−/− adult heart may lead to effects on downstream signaling pathways important for regulating heart remodeling, including the oxytocin-natriuretic peptide system, which is known to be responsive to ATRA. This possibility contributes to the cardiac phenotype observed in Bco1−/− mice.

Differences in lipid accumulation and metabolism could also directly contribute to the observed impairments in Bco1−/− heart function. Young et al. (50) established that increased fatty acid availability is associated with lipid accumulation and cardiac contractile dysfunction in obese Zucker rats. This was extended by Zhou et al. (51), who demonstrated that increased fatty acid availability was associated with elevated heart ceramide levels. Finck et al. (15) reached the same conclusion studying mouse models. We observed elevated levels of both FFAs and ceramides in the hearts of Bco1−/− mice. Interestingly, ATRA treatment of cultured human SK-N-SK and SK-N-AS neuroblastoma cells or MCF-7 mammary epithelial cells results in increased cellular ceramide levels (8, 22). For these cell model systems, this caused an inhibition of cell growth. Thus, there is precedence in the literature for ATRA modulation of endogenous ceramide concentrations, which then affects cell growth. Collectively, this leads to the hypothesis that the altered retinoid homeostasis in the Bco1−/− heart affects tissue FFA availability and ceramide concentrations, which contribute to the observed impaired cardiac contractility. Although this hypothesis, and/or the other two hypotheses discussed above, could explain the metabolic and contractility phenotypes we observed for hearts of Bco1−/− mice, this will need to be established through future studies aimed specifically at understanding better these possibilities.

**REFERENCES**


