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Ablation of β,β-carotene-9′,10′-oxygenase 2 remodels the hypothalamic metabolome leading to metabolic disorders in mice☆

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Abstract

β,β-Carotene-9′,10′-oxygenase 2 (BCO2) is a protein localized to the inner membrane of mitochondria. It was initially discovered as an enzyme that catalyzes the asymmetric cleavage of carotenoids. Systemic depletion of BCO2 causes increased food intake and impaired hepatic lipid metabolism in mice. The aim of this current study was to determine the extent to which BCO2 exerts its role in hypothalamic nutrient metabolism and feeding behavior through remodeling the hypothalamic metabolome in mice. Male BCO2 knockout (KO) and the isogenic wild-type 129S6 (WT) mice at 6 weeks of age were used for metabolic and cytokine and hypothalamic metabolomics and biochemical analysis. Compared to the WT, BCO2 KO mice exhibited widespread disruptions in metabolism and metabolite homeostasis, an increase in fasting blood glucose, a decrease in circulating glucagon and leptin, an elevation of plasma interleukin 1 beta and tumor necrosis factor alpha, and impaired AMP-activated protein kinase signaling. The global hypothalamic metabolomic results revealed that depletion of BCO2 resulted in striking metabolic changes, including suppression of long-chain fatty acids transport into mitochondria, inhibition of the metabolism of dipeptides and sulfur-containing amino acids, and stimulation of local oxidative stress and inflammation in the hypothalamus of BCO2 KO mice. These findings suggest that BCO2 regulates hypothalamic mitochondrial function, nutrient metabolism, and local oxidative stress and inflammation. Complex interplay between the hormone signaling and impaired lipid and glucose metabolism could account for initiation of oxidative stress, inflammation and eventual metabolic disorders in BCO2 KO mice.
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1. Introduction

The excess of food intake is a major contributor to the high incidence of metabolic disorders, such as obesity and diabetes, which have emerged as serious health issues around the world. Such feeding behavior requires a coordinated effort of neurotransmitters, peptides and hormones in various nuclei throughout the brain [1]. The hypothalamus is the key region of the brain involved in modulating feeding behavior through signal integration from both central and peripheral pathways. Two functionally distinct populations of neurons, which express anorexigenic proopiomelanocortin (POMC) or neuropeptide Y/orexigenic agouti-related protein (NPY/AgRP), respond to a variety of circulating hormones to regulate both short- and long-term energy homeostasis [2]. Leptin inhibits NPY/AgRP neurons and activates POMC neurons to suppress food intake, enhance lipolysis, decrease lipogenesis and stimulate energy expenditure. Insulin functions to promote energy utilization and/or storage through the regulation of glucose uptake and the metabolism of fatty acids in response to increased plasma glucose. POMC and NPY/AgRP neurons also respond to peripheral gut hormones, such as ghrelin and glucagon-like peptide-1 (GLP-1), to modulate feeding behavior [3].

Abbreviations: ACC, acetyl-CoA carboxylase; AgRP, agouti-related protein; AKT, protein kinase B; AMPK, AMP-activated protein kinase; BCAA, branched-chain amino acid; BCO2, β,β-carotene-9′,10′-oxygenase2; CPT, carnitine palmitoyltransferase; GCS, glutamyl cysteine synthetase; GGT, gamma-glutamyl transpeptidase; GLP-1, glucagon-like peptide-1; GS, glutathione synthetase; GSH, glutathione; HMG, 3-hydroxy-3-methylglutarate; IL-18, interleukin 18; IL-1β, interleukin 1β; IPA, Ingenuity Pathway Analysis; IRS2, insulin receptor substrate 2; LCFA, long-chain fatty acid; LepR, leptin receptor; NLRP1, NLR family pyrin domain containing 1; NPY, neuropeptide Y; OPH, ophthalmate; POMC, proopiomelanocortin; PPAR-α, peroxisome proliferator activated receptor α; PTT, pyruvate tolerance test; SAM, *S*-adenosyl methionine; TNF-α, tumor necrosis factor alpha.

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Nutrients, such as carbohydrates, amino acids and fatty acids, can function as signaling molecules and are detected by the hypothalamus to modulate feeding behavior [4]. Glucosensing neurons regulate their membrane potential, firing rate and ion channel function in response to ambient glucose levels. Glucosensing is especially important in stimulating appetite when there is a reduction in glucose availability [5]. Some amino acids, especially branched-chain amino acids (BCAAs), may also produce signals relevant to food intake and protein balance [6]. Increasing evidence indicates that fatty acid sensing in the brain is another vital regulator of feeding behavior, energy utilization and storage [7,8]. Both the peripheral and central administration of fatty acid synthase inhibitors significantly suppressed appetite [9,10]. Whereas glucose and fatty acids are the brain's primary fuel sources that generate ATP through oxidative metabolism in hypothalamic mitochondria, the role of the hypothalamic mitochondria in feeding behavior and energy metabolism has not been fully appreciated.

β,β-Carotene-9′,10′-oxygenase 2 (BCO2) is a protein localized to the inner membrane of mitochondria [11]. It was initially identified as an enzyme that catalyzes the asymmetric cleavage of carotenoids [12–14]. Yet, the enzymatic function of BCO2 in the retina of primates is a topic of debate due in part to the absence of a correlation between BCO2 activity and retinal carotenoid levels in humans [15,16]. Genetic studies have revealed that BCO2 mutations are linked to alterations in interleukin 18 (IL-18) production, insulin resistance, obesity, macular degeneration and prostate cancer in humans [17–20]. Our laboratory and others have reported that intact BCO2 is essential to hepatic mitochondrial function and lipid metabolism in laboratory animals [12,21–28]. Our most recent findings show that $BCO2^{-/-}$ knockout (KO) mice exhibit higher food intake [21]. The aim of this study was to investigate the metabolic mechanism by which BCO2 regulates hypothalamic mitochondrial function and feeding behavior by targeting the hypothalamic metabolome and relevant signaling pathways in energy homeostasis.

2. Methods

2.1. Animals, animal care and fasting blood glucose test

Male wild-type 129S6 (WT) (Taconic, Germantown, NY, USA) and $BCO2^{-/-}$ KO mice at 6 weeks of age were used in this study [21]. Of note, those BCO2 KO mice were backcrossed for seven generations to a WT genetic background and were considered to be isogenic with 129S6 mice from Taconic, although some differences between the mouse strains could still be attributed to genetic background, according to the definition of inbred mice by the Jackson Laboratory. Mice had free access to water and food throughout the study. All animal experiments and procedures were performed in accordance with our protocols approved by the Oklahoma State University Institutional Animal Care and Use Committee (protocol # HS-13-4 and HS-14-4). Mice were group housed (three mice/cage) in a controlled environment with a 12-h light/dark cycle and fed a standard chow diet (Catalog # D12450B; Research Diets, Inc., New Brunswick, NJ, USA). The macronutrient composition of the diet is 10% kcal from fat, 20% kcal from protein and 70% kcal from carbohydrate. The diet contained sufficient amounts of vitamins (primary vitamin mix V10001 and choline bitartrate) and minerals (primary mineral mix S10026, dicalcium phosphate and potassium citrate) as recommended for rodents [21]. The amounts of carotenoids (<1 μg/g) were below the detectable levels as measured by high-performance liquid chromatography (HPLC) in both the diet and mouse liver tissues from the WT and BCO2 KO mice. However, the HPLC analyses (μg/g detection limit for carotenoids) did not completely exclude a contribution of carotenoids to the phenotype of BCO2 KO mice due to the fact that there might be some trace amounts of carotenoids in those mice. At study termination, mice were fasted for 5–6 h prior to sacrifice by CO_2 followed by cervical dislocation according to the approved protocols. Blood was collected into EDTA-coated tubes for the collection of plasma. Hypothalamic tissues were collected for metabolomics and other experiments. Before necropsy, fasting blood glucose was tested by tail snip using the Precision Xtra blood glucose monitoring system (Alameda, CA, USA).

2.2. Plasma parameter measures

Circulating leptin, insulin, glucagon, IL-18, interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α) were assessed using the Bio-Plex mouse diabetes panel and the cytokine panel assay kits (BIO-RAD Laboratories, Hercules, CA, USA) according to the manufacturer's instruction.

2.3. Hypothalamic mitochondrial respiratory activity

2.3.1. Hypothalamic mitochondrial isolation

After euthanization, hypothalamic tissues were immediately harvested to assess mitochondrial function as described previously [21]. Briefly, the tissue was minced in 0.5-ml mitochondrial isolation buffer at pH 7.2 (210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L HEPES, 1 mmol/L EGTA and 0.5% [w/v] fatty-acid-free bovine serum albumin) and homogenized (Qiagen tissueruptor, Cat. No. 9001271, Limburg, CA, USA) for 10 s at the lowest speed on ice. The homogenate was centrifuged at 600×*g* for 5 min at 4°C, and then the supernatant was centrifuged at 5000×*g* for 5 min at 4°C to collect mitochondrial pellets. Mitochondrial pellets were resuspended in 1-ml mitochondrial assay solution (1X-MAS, pH 7.2: 220 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L $MgCl_2$, 2 mmol/L HEPES, 10 mmol/L KH_2PO_4, 1 mmol/L EGTA, 0.2% [w/v] fatty-acid-free BSA). Mitochondrial fraction protein concentrations were determined using a method of BCA [21]. The distribution of BCO2 proteins in mitochondria of hypothalamic tissues was confirmed by Western blot, with expression of hepatic mitochondrial BCO2 proteins serving as a positive control (Supplemental Fig. S1A).

2.3.2. Mitochondrial respiration complex II activity

Freshly isolated mitochondria were then used to assess cellular respiration using an electron flow assay to determine the mitochondrial respiratory activity in the Seahorse Extracellular Flux Analyzer (Agilent-Seahorse Bioscience, North Billerica, MA, USA), as reported previously [21]. The oxygen consumption rate (OCR) difference before and after antimycin A administration reflected complex II capacity. The OCR value was expressed as pmol O_2/μg protein/min.

2.4. Immunoblotting analysis

Proteins were extracted from hypothalamic tissues with a lysis buffer containing 20 mmol/L Tris–HCl (pH 7.5), 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 0.5% (v/v) Triton X-100 and 0.1% (v/v) protease and phosphatase inhibitor cocktails, respectively (Sigma-Aldrich, St. Louis, MO, USA). Immunoblot analysis was conducted as previously described [21,22]. Total pixel intensity of each protein band was normalized to a loading control for graphing and statistical analysis. Antibodies against BCO2 (catalog # 14324-1-AP), cytochrome C (catalog # 10993-1-AP) and leptin receptor (LepR, catalog # 20996-1-AP) were purchased from ProteinTech Group (Chicago, IL, USA). Antibodies against β-actin (catalog # 4967), insulin receptor substrate 2 (IRS2, catalog # 4502), protein kinase B (AKT, catalog # 2961), phospho-Thr308-AKT (catalog # 2965), AMP-activated protein kinase α (AMPKα, catalog # 2603), phospho-Thr172-AMPKα (catalog # 2535), AMPKβ1/2 (catalog # 4150), phospho-Ser108-AMPKβ1/2 (catalog # 4181) and IL-1β (catalog # 12426) were obtained from Cell Signaling Technology (Danvers, MA, USA). NLR family pyrin

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