THE IMPACT OF B, B-CAROTENE - 9',10'-OXYGENASE2 (BCO2) ON MITOCHONDRIAL FUNCTION AND HYPOTHALAMIC METABOLISM

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Abstract: β,β-carotene-9',10'-oxygenase2 (BCO2) is a carotenoid cleavage enzyme localized to the inner membrane of mitochondria. Hypothalamus is the key brain region that regulates energy homeostasis through nutrient sensing and signal integration from central and peripheral pathways. The aim of this study is to explore the impact of BCO2 on maintaining mitochondrial function and hypothalamic metabolism, as well as the mechanism of why BCO2 $^{-/-}$ mice exhibit orexigenic phenotype. Using 129S6 wild type (WT) and BCO2 $^{-/-}$ mice model, we measured metabolic profiling differences in hypothalamus caused by the deletion of the BCO2 gene. Mitochondrial proteomic and functional analyses were also conducted to evaluate the impact of BCO2 on mitochondria. Metabolomics results revealed striking metabolic changes between genotypes. The top ranking metabolites in distinguishing WT and BCO2$^{-/-}$ mice suggested key differences in transport of long chain fatty acids into mitochondria, biosynthesis of bile acids; metabolism of sulfur-containing amino acids; and markers of inflammation. The significantly lower long chain fatty acids and glucose in the hypothalamus may regulate energy homeostasis by altering nutrient sensing. Within the mitochondria, altered pathways including the proteins involved in Krebs cycle, electron transport chain and fatty acids β-oxidation were observed in hypothalamic mitochondria of BCO2 $^{-/-}$ mice. The deletion of BCO2 also led to the significantly decreased capacity of complex II of electron transport chain. In conclusion, the absence of BCO2 may be closely associated with hypothalamic metabolism disorder and mitochondrial dysfunction caused by the perturbed energy utilization. The significantly lower leptin level and lower hypothalamic glucose and lipids sensing may be the major contributor to the enhanced appetite in BCO2$^{-/-}$ mice.

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CHAPTER I

INTRODUCTION

Obesity has emerged as one of the most serious health issues in the world, which is closely related to type-2 diabetes [1], neurodegenerative diseases [2], cardiovascular disease [3], and some forms of cancer [4, 5]. Obesity is the result of energy intake exceeding energy expenditure. It is a disorder of energy balance [6] commonly caused by a combination of excessive food intake, lack of physical activity, and genetic susceptibility [7].

The hypothalamus is a key region in the brain for modulating feeding behavior and energy expenditure through nutrient sensing and signal integration from central and peripheral pathways [8-10].Two populations of neurons in the arcuate nucleus (ARC) of the hypothalamus play an important role in maintaining energy balance. One population of neurons express anorexigenic proopiomelanocortin (POMC) and the other express orexigenic agouti-related protein (AgRP)/neuropeptide Y (NPY). POMC and NPY/AgRP neurons could respond to leptin and insulin and interact with gut hormones such as ghrelin, glucagon-like peptide-1 (GLP-1), peptide YY3–36, cholecystokinin, and pancreatic polypeptide to maintain energy balance [10, 11].

Hypothalamic mitochondria are involved in the modulation of energy balance by mitochondrial dynamic to modulate AGRP and POMC neuronal activity [12, 13]. Mitochondria could change their number and size by fusion and fission to adapt to the energy environment [12]. It was reported that mitochondria could fuse in AgRP neurons, and fission in POMC neurons during a positive energy balance to enable sustained neuronal activity and maximize the uptake of energy. The morphology of mitochondria also differs between obese and lean mice. Reduced mitochondrial length and loss of mitochondria-endoplasmic reticulum (ER) interactions were observed in diet-induced obese mice compared to lean controls [13].

The mitochondria are responsible for most of the ATP production in the cell through the electron transport chain (ETC)/oxidative phosphorylation (OXPHOS). These processes occur in the inner membrane of mitochondria. The ATP producing capacity of mitochondria is another factor that affects energy homeostasis. Reduced ATP level in the hypothalamus leads to persistent AMPK stimulation. Leptin could function through inhibition of hypothalamic AMPK signaling to increase energy expenditure and lower food intake. Therefore, sustained AMPK stimulation requires a high level of leptin to suppress the effects of AMPK on AgRP and NPY [14]. Such impairment in ATP formation would lead to the tendency of eating more than what the body needs and conserving energy by lowering energy expenditure.

BCO2 (β, β-carotene-9',10'-oxygenase2) is an enzyme that catalyzes the asymmetric cleavage of carotenoids in the inner membrane of mitochondria [15]. Recent controversial research findings demonstrated that BCO2 may not be activated in the human macula where carotenoids preferentially accumulate [16, 17], suggesting that BCO2 may not only function as a carotenoid cleavage enzyme which warrants further investigation. For example, BCO2 is involved in inflammation by regulating the transcription of the IL-18 gene [18]. It also

participates in macular degeneration in humans [16] and in the process of anemia and apoptosis of red blood cells in zebrafish [19]. Furthermore, BCO2 protein expression is reduced in obese [20] and diabetic mice [21]. However, how BCO2 impacts these processes, whether in a direct or an indirect manner, is still unknown.

The expression and activity of BCO2 have been verified in humans [22], mice [23], rats [24], cattle [25], chickens [26], and sheep [27]. BCO2 is expressed in many types of cells, such as liver hepatocytes, epithelial cells of villi and endothelial cells in the duodenum, and other tissues such as kidney and lungs [28-30]. Recently, BCO2 was found to be in the inner mitochondrial membrane [31], where complexes of the ETC/OXPHOS are located. In accordance with this finding, carotenoids were found to be accumulated in the mitochondria of BCO2 knockout mice, which further led to mitochondrial dysfunction, indicated by an increased manganese superoxide dismutase (MnSOD) level and decreased respiratory activity [23]. Given that the key position of the hypothalamus in energy balance and the potential role of BCO2 in mitochondrial function, BCO2 may play a novel function in maintaining metabolic homeostasis in the hypothalamus. Therefore, we hypothesize that BCO2 contributes to hypothalamic mitochondrial function through its impact on the ETC/OXPHOS. Complete ablation of BCO2 will elevate oxidative stress, perturb energy metabolism, and impair cell signaling in hypothalamus. The following four specific objectives have been developed to test the hypothesis.

Objective 1: To characterize the impact of BCO2 on blood parameters, such as fasting glucose, lipid profile and circulating leptin, insulin and glucagon level;

Objective 2: To elucidate the impact of BCO2 on the hypothalamic global metabolic status;

Objective 3: To profile the change in hypothalamic mitochondrial proteome in wild-type (WT) and $BCO2^{-/-}$ mice; and

Objective 4: To determine the effect of BCO2 on hypothalamic mitochondrial function

(basal respiratory activity, proton leak, ATP production, and capacity of ETC complexes) between WT and $BCO2^{-/-}$ mice.

CHAPTER II

LITERATURE REVIEW

This literature review includes an overview of the energy homeostasis at the hypothalamic and mitochondrial level, as well as the role of BCO2 in mitochondrial function.

1. Predominant role of hypothalamus in mediating energy homeostasis

Obesity refers to the metabolic state in which excess fat is accumulated in peripheral tissues, such as white adipose tissue, liver, and muscle [32]. Obesity, which has negative effects on both the quality and length of life, is commonly caused by a combination of excessive food intake, lack of physical activity, and genetic susceptibility [7, 33]. The hypothalamus plays a significant role in controlling energy expenditure and food intake, which are the determinants of metabolic phenotype.

The hypothalamus is organized into anatomically discrete nuclei: paraventricular nucleus (PVN), lateral hypothalamic area (LHA), dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamus (VMH), and ARC [34]. These neuronal clusters form interconnected neuronal circuits to sense information inputs from the process of eating, ingestion, absorption, and metabolism, as well as the change of energy storage [34]. Early hypothalamic lesion

experiments showed that LHA acts as the "hunger center" and is associated with hypophagia and loss of body weight, while the VMH is the 'satiety center' and is linked to hyperphagia and obesity [35]. The ARC is located in the basal part of the hypothalamus and is in close contact with the median eminence. The ARC could sense fluctuations in signals from hormones, nutrients, and other molecules that are transported within the blood [8]. Thus the ARC has been postulated to play a fundamental role in sensing the global energy status of the organism [8, 9] and integrating signals from central and peripheral pathways [10].

The POMC and AgRP/NPY are expressed by two key populations of neurons that have opposite effects on appetite in the ARC. POMC neurons exert an anorexigenic effect by co-expressing POMC and cocaine- and amphetamine-regulated transcript (CART) [34]. POMC is the precursor of α-melanocyte stimulating hormone (α-MSH), which could activate melanocortin 4 receptors (MC4R). Activation of MC4R would ultimately inhibit appetite and increase energy expenditure [36]. The other population of neurons co-express orexigenic NPY and AgRP. NPY exerts an orexigenic effect via different subtypes of NPY receptors on downstream neurons, while AgRP could directly block α-MSH action [36]. Both of these populations of neurons act on the PVN, and the ARC also interacts with other hypothalamic nuclei such as the LHA, VMH, and DMH [37].

1.1. Hypothalamus regulates energy homeostasis via circulating hormones

NPY/AgRP and POMC/CART neurons respond to a variety of circulating hormones to regulate energy balance. Insulin and leptin are the two most critical anorexigenic hormones that are involved in energy homeostasis [38]. The activation of leptin and insulin are mediated by their receptors in the central nervous system (CNS). Leptin receptor (LepR) and insulin receptor (InsR) are highly expressed in the AgRP and POMC neurons of the ARC [39].

Leptin is a hormone secreted from white adipose tissue and circulates through the body in proportion to body fat mass. It plays a major role in energy homeostasis by inhibiting food intake, enhancing lipolysis, decreasing lipogenesis, and elevating energy expenditure. Leptin acts via LepR in the hypothalamus (predominantly the VMN and ARC, but also the choroid plexus) to regulate energy balance and neuroendocrine function [40-42]. The POMC neurons are activated by leptin, resulting in α-MSH release from POMC axon terminals. The α-MSH could further activate MC4R to increase energy expenditure and suppress food intake [43]. At the same time, the activity of NPY/AgRP neurons is inhibited by leptin [44]. Otherwise, it would antagonize the effect of α-MSH on MC4Rs through the release of AgRP [45]. Besides the actions of leptin via the brain, energy expenditure and food intake could also be modified through the direct action of LepRs on a peripheral target, including the β cells of the pancreas, liver, muscle, and fat [46].

Insulin is secreted by pancreatic β-cell in response to increased plasma glucose levels. It promotes energy utilization and storage through the regulation of glucose uptake and the metabolism of fatty acids. In skeletal muscle, insulin promotes glucose uptake by stimulating the movement of intracellular vesicles containing GLUT4 from cytosol to the plasma membrane, which increases the transport capacity for glucose. In adipose tissue, insulin reduces free fatty acid (FFA) efflux from adipocytes, thus decreasing lipolysis and facilitating fat storage. Furthermore, insulin promotes the storage of glucose as glycogen and/or triacylglycerol in the liver and inhibits hepatic glucose production by decreasing the activity of gluconeogenic enzymes [47].

Hypothalamic leptin and insulin signaling are involved in both short- and long-term energy regulation (**Fig. 1** (revised from [9])). Janus kinase (JAK) is recruited and activated when leptin binds to the extracellular domain of LepR-b. The activated JAK in turn helps to phosphorylate LepR-b [48]. During this process, the signal transducer and activator of

transcription 3 (STAT3) are activated. The phosphorylated STAT3 could stimulate POMC and inhibit AgRP [49]. The activation of STAT3 is associated with the elevated expression of the suppressor of cytokine signaling 3 (SOCS3). SOCS3 will bind with the LepR-b-JAK-2 complex, thus inhibiting leptin signaling [50]. Insulin signaling is initiated by insulin binding to the α subunit of InsR [51]. The conformational change of the α subunit exhibits intrinsic tyrosine kinase activity, which could activate InsR [52]. The insulin receptor substance (IRS) is activated through the phosphorylation process resulting from InsR. The leptin signaling and insulin signaling converge together through the phosphoinositide 3-kinase (PI3K) – Phosphatidylinositol 3,4,5-trisphosphate (PIP_3)– 3-phosphoinositide-dependent protein kinase 1(PDK1) – forkhead box protein O1 (FoxO1) signaling pathway [9]. The PI3K, which is activated by leptin or IRS, could promote the synthesis of PIP3 [53]. As the PIP3 accumulates, PDK1 is activated, which leads to the activation of protein kinase B (PKB, also known as AKT). Within the nucleus, FoxO1 could activate AgRP and inhibit POMC expression [54]. Activation of AKT induces the phosphorylation of FoxO1 and further leads to the export of FoxO1 into the cytoplasm. This would facilitate STAT3 to bind with POMC/AgRP. AKT also functions to activate the mammalian target of rapamycin (mTOR), and the activation of mTOR involved in phosphorylation and inhibition of adenosine monophosphate (AMP)-activated protein kinase (AMPK). Both mTOR and AMPK act as sensors of the nutrient status that is involved in the regulation of food intake and energy expenditure, which will be discussed below.

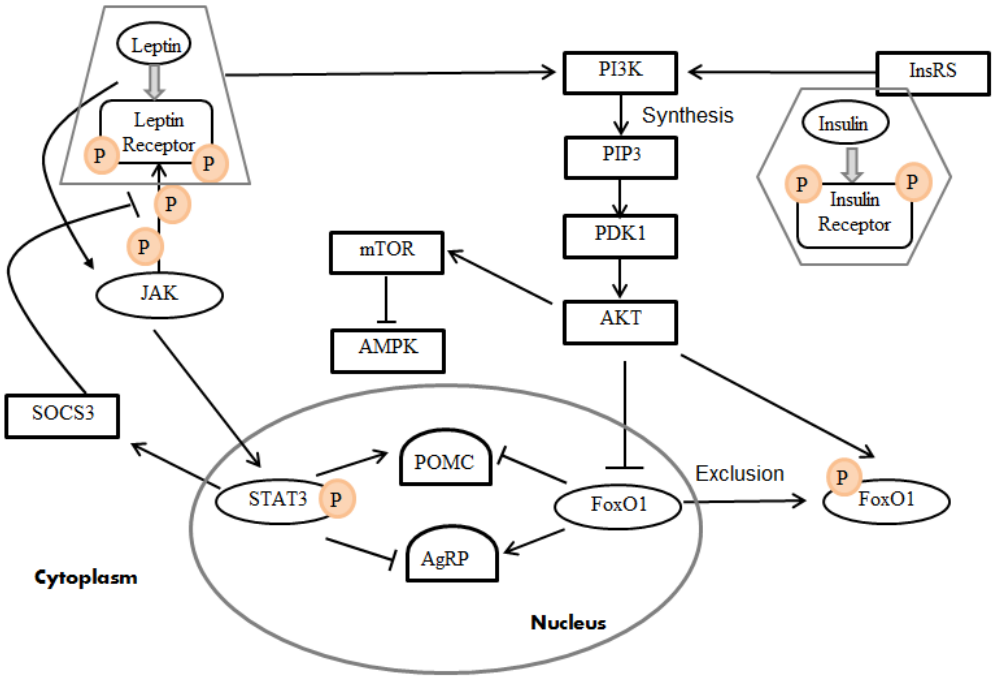


Fig. 1 Leptin and insulin signaling pathway in the hypothalamus (Revised from [9]) **.** Leptin and insulin are secreted from white adipose tissue and the pancreas, respectively. Leptin and insulin activate their receptors in the hypothalamus. Both of the activation of LepR-b and InsR could further activate PI3K–PIP3–PKD1–FoxO1 signalling pathway. Phosphorylation of LepR-b by JAK2 also leads to the recruitment and phosphorylation of STAT3. SOCS3 would desensitize leptin signaling by suppressing STAT3 activation.

Most obese patients have high serum leptin levels leading to leptin resistance. Similarly, insulin resistance is characterized by a decreased ability to respond to insulin. In most cases, impaired insulin sensitivity of the target organs, rather than the low insulin level in the body, eventually leads to type 2 diabetes. Activation of the IκB kinaseβ (IKK), c-Jun N-terminal kinase (JNK), and protein kinase C iota (PKCτ) signaling pathways and increased expression of SOCS3 and PTP1B are the major molecular mechanisms involved in the induction of hypothalamic leptin resistance and insulin resistance [55-59].

The hypothalamus can also interact with a variety of gut hormones. For example, peptide YY3–36, cholecystokinin, GLP-1, oxyntomodulin (OXM), and pancreatic polypeptide act synergistically to suppress appetite, whereas ghrelin acts to stimulate food intake [10, 11]. By relaying signals of nutrient level and energy status in the gut to the CNS, the interaction between NPY/AgRP and POMC/CART neurons and gut hormones plays a critical role in regulating food intake.

1.2. Hypothalamus regulates energy balance via sensing nutrient status

Aside from the signals from hormones, the hypothalamus is also sensitive to circulating metabolites. Two types of glucosensing neurons are either inhibited or excited as glucose level fluctuates [60]. Glucosensing neurons regulate their membrane potential, firing rate, and ion channel function to sense ambient glucose. Glucose sensing is especially important in stimulating appetite when glucose availability drops [61-63].

Circulating lipids such as triglyceride and long-chain fatty acids (LCFAs) function similar to insulin through elevation of the LCFA-CoA level in the hypothalamus [64]. LCFA gets into the brain cells by passive diffusion [65] or translocation via a carrier protein [66]. In the diffusion model, fatty acids diffuse across the luminal and transluminal leaflets of endothelial cells and plasma membrane of neural cells [65, 67]. For the protein mediated transport model,

protein transporters expressed on cell membrane, such as fatty acid tanslocase/CD36, fatty acid transport protein (FATP) 1-6, and plasma membrane fatty acid binding protein are involved in the transport of fatty acid into the blood brain barrier [66, 68, 69]. Upon entry into the neurons, LCFA is esterified into LCFA-CoA via Acetyl-CoA synthetase (ACS). LCFA-CoA signals nutrient abundance and exhibits insulin-like effects in the brain, including the hypothalamic ARC, to modulate energy homeostasis [70]. Brain fatty acids act as a satiety signal to inhibit appetite [71]. Both the peripheral and central administration of a fatty acid synthase inhibitor significantly suppresses appetite [72, 73]. An *in vivo* study showed food intake and hepatic glucose production were inhibited after three days of intracerebroventricular infusion of oleic acid [74]. The accumulation of LCFA-CoA within specific CNS neurons could activate catabolic neural pathways to inhibit appetite and hepatic glucose production [75]. The anorectic effect of LCFA-CoA accumulation may lie downstream of the leptin-induced inhibition of hypothalamic AMPK [76]. LCFA-CoA in the brain activates protein kinase C and further phosphorylates and activates ATP-sensitive potassium (K_{ATP}) channels. This lipid-sensing pathway in the hypothalamus activates N-methyl-d-aspartate (NMDA) receptor to trigger the hepatic vagus nerve to regulate glucose production. In this way, neuronal signals are transmitted via the hepatic vagus nerve to the liver.

In contrast to intracellular LCFA-CoA accumulation, which is a sensor of nutrient abundance, the AMPK is a sensor of nutrient insufficiency. At the peripheral level, AMPK activation leads to increased oxidation to maintain cellular energy availability. In the ARC, AMPK activation results in increased food intake and a trend to conserve energy [77]. When cells sense the low fuel availability indicated by an increased AMP/ATP ratio, the activation of AMPK enhances substrate oxidation to replenish the depleted ATP levels [70]. Research conducted in mice showed that within the mediobasal hypothalamus, activation of AMPK increased food intake and body weight, while inhibition of AMPK led to the opposite results [77]. Hypothalamic

AMPK activity is inhibited by glucose, insulin, and leptin and stimulated by ghrelin [77, 78]. The change in AMPK signaling may also alter the appetite governed by these hormones. A consequence of increased AMPK activity is the oxidation of LCFA-CoA, which might induce orexigenic effects. Furthermore, AMPK could inhibit the activity of mTOR, another nutrient sensor, which will be discussed below.

mTOR is an evolutionarily conserved serine-threonine kinase involved in the regulation of cell-cycle progression and growth via sensing energy status [79]. It was demonstrated that in peripheral tissues, mTOR participates in coupling cellular energy status and facilitated cellular anabolic processes, such as protein synthesis in response to growth factors, amino acids, glucose, and stress [10]. Later, research found that mTOR was also expressed in the hypothalamus and play an important role in hypothalamic regulation of energy homeostasis in response to nutrient availability. Cota et al. found that administration of leucine in rats led to elevated hypothalamic mTOR signaling and decreased food intake, as well as body weight [79]. They also showed that leptin could activate hypothalamic mTOR activity and that inhibition of mTOR signaling would block the leptin-induced suppression of food intake [79]. Both mTOR and AMPK represent a potential site of convergence for both hormonal and nutrient sensing.

2. The role of mitochondria in maintaining cellular energy metabolism

We have discussed that the hypothalamus is the most important organ to modulate energy homeostasis in brain. When we take a closer look at the cell organelle level, the mitochondrion is the key organelle that maintains cellular energy metabolism. The mitochondrion is a double-membrane bound organelle in most eukaryotic cells [80]. It is composed of an outer membrane, intermembrane space, inner membrane, and the matrix. Variety of enzymes are involved in different metabolic processes in the mitochondrial matrix. One major role of a mitochondrion is to produce energy through the tricarboxylic acid (TCA) cycle and ETC/OXPHOS. In addition to

the utilization of the carbohydrate-derived substrate, the β-oxidation of fatty acids is another resource to generate ATP that mainly takes place in the mitochondria. Besides providing a platform for the oxidation of fuel substrates, mitochondria are also involved in a variety of cell signaling pathways by acting as transducers and effectors in the process of cell death, innate immunity, and autophagy [81, 82]. Furthermore, mitochondria also serve as calcium buffers and sources of free radicals [83, 84].

2.1. Hypothalamic mitochondria and energy homeostasis

Hypothalamic mitochondrial dynamic is involved in the energy homeostasis through the regulation of AgRP and POMC neuronal activity [12, 13]. Mitochondrial dynamics refer to the dynamic fission and fusion behavior and morphology change of mitochondria [85] in response to the environment, especially to the energy status. Mitofusins (Mfn), namely Mfn1 and Mfn2, work coordinately to regulate mitochondrial fusion. The depletion of Mfn1 and Mfn2 in AgRP neurons of mice led to decreased activity of the AgRP neuron, which was accompanied by weight loss. [12]. When mice were deprived of food, an increased number and decreased size of mitochondria were observed in AgRP neurons [12]. When fasted mice were exposed to excess food, the number of mitochondria decreased but their size increased in the AgRP neurons. However, the opposite fission-like dynamic phenomenon was observed in the POMC neurons, which exhibited reduced mitochondrial density and coverage [12]. Similarly, mitochondria in the AgRP neurons were fused when mice were fed a high-fat diet. These findings indicate that mitochondria could fuse in AgRP neurons and fissure in POMC neurons during a positive energy balance to enable sustained neuronal activity and maximize the uptake of energy.

The morphology of mitochondria also differs between obese and lean mice. It has been reported that mitochondrial networks and mitochondria-endoplasmic reticulum (ER) contacts in POMC are important for energy homeostasis [13]. Mitochondria are found as thread-like, tubular

organelles that often branch and form connections with each other through tubular reticulum [86]. Such branched structure of the mitochondrial network is constituted by separate mitochondria [87]. Decreased mitochondrial length and branching was observed in diet-induced obese mice compared to lean controls. Diet-induced obese mice also showed a significant reduction of mitochondria-ER contacts in POMC neurons.

2.2. Mitochondria and adipocyte transcription factors

Some adipocyte transcription factors play a critical role in modulating mitochondrial function and biogenesis [88]. The peroxisome proliferator-activated receptor gamma coactivator (PGC) family is a group of transcriptional coactivators. A reduced PGC-1α level was observed in the adipose tissues of obese patients [89] and in genetically or diet-induced obese mice [90]. PGC-1α and PGC-1β not only participate in the regulation of glucose metabolism, fatty acid metabolism, and lipid accumulation, but also function to affect the expression of the mitochondrial biogenesis gene and are involved in the development of obesity [91]. PGC-1 stimulates mitochondrial biogenesis and respiration in myotubes of muscle via an induction of uncoupling protein 2 (UCP2), and through the regulation of the nuclear respiratory factors (NRFs). Deficiency in either PGC-1α or PGC-1β in preadipocytes of brown adipose tissue (BAT) led to impaired mitochondrial gene expression, density, and respiratory activity [92] .

PR Domain Containing 16 (PRDM16) is the coactivator of PGC-1α and PGC-1β, which is important for mitochondrial biogenesis and uncoupled cellular respiration [93, 94]. Decreased PRDM16 expression in BAT leads to the inhibition of mitochondrial gene expression and stimulation of myogenic markers [94, 95]. Another important transcriptional coactivator is bone morphogenetic protein 7 (BMP7), a subgroup of the transforming growth factor beta (TGF-β) superfamily, which increases mitochondrial density and the expression of mitochondrial

biogenesis genes through activation of p38 mitogen-activated protein kinases (MAPK) and PGC-1α [96].

3. Mitochondrial dysfunction in the development of obesity

A renaissance in mitochondrial research has occurred during the past decade. Besides its function in energy production, mitochondria participate in many processes including cell signaling, cell differentiation, and apoptosis [87]. Mitochondria are also involved in the generation of reactive oxygen species (ROS), ER stress, and inflammation. Mitochondrial dysfunction is thought to trigger many chronic diseases, including neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease, liver and kidney disorders, diabetes, and the aging process [97]. By integrating metabolic information including ATP level, oxidative stress, ER stress, inflammation, and cell signaling, mitochondria play an important role during the development of obesity.

3.1. Inadequate ATP production and obesity

A possible source of leptin resistance is impaired mitochondrial ATP production. Research has shown that altered mitochondrial energy production, especially in skeletal muscles, is a major factor to disrupt a chain of metabolic events leading to obesity [14]. In the hypothalamus, the reduced energy producing capacity of mitochondria led to persistently high hypothalamic AMPK stimulation. Leptin could function through inhibition of hypothalamic AMPK signaling to increase energy expenditure and lower food intake. Therefore, sustained AMPK stimulation requires a high level of leptin to suppress the regulation of AMPK on AgRP and NPY [14]. Such impairment in ATP formation would lead to the tendency of eating more than what the body needs and conserving more energy by lowering energy expenditure.

3.2. Oxidative stress and obesity

In the mitochondria, energy is produced from the TCA cycle, ETC/OXPHOS, and β-oxidation of fatty acids. Approximately 90% of oxygen in the cell is consumed by the mitochondria [98]. The mitochondrial respiratory chain is one of the major sources of ROS. While mitochondria generate ATP through ETC/OXPHOS, ROS is generated as a byproduct [99]. The electrons are transported from NADH and FADH2 to complex III, and then to complex IV. Eventually, electrons are passed on to oxygen, yielding H_2O. However, some electrons might escape from ETC. Once these electrons leak into the mitochondrial matrix, the incomplete electron transfer will lead to the formation of superoxide ($O_2^{-}\cdot$). It has been reported that complex I is a major donor of electrons to generate ROS in the brain [100]. Even under normal physiological conditions, approximately 1–5% of the O_2 consumed by mitochondria is converted to ROS [87]. ROS refer to a variety of oxygen free radicals, such as superoxide anion radical ($O_2^{-}\cdot$), hydroxyl radical ($\cdot OH$), and non-radical oxidants, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [101]. Most of the intracellular ROS are derived from superoxides.

ROS are important for various cell functions and act as a mediator of intracellular signaling cascades. However, excessive production of ROS may cause damage to cells and ultimately lead to the apoptosis or necrosis of the cells [102]. Organisms need to maintain equilibrium between the ROS produced during the energy generating process and the damaging effects of ROS, which is known as oxidative stress [87]. Oxidative stress is usually associated with the etiology of obesity, type 2 diabetes, cancer, and many other chronic diseases [103, 104]. In the cells, there are various anti-oxidative enzymes, such as superoxide dismutase, copper/zinc superoxide dismutase, glutathione peroxidase, and catalase, to deal with the continuous production of ROS [105].

The excess lipid accumulation in obese individual represents an excess of energy, but obese individuals fatigue easily and show lower physical endurance, reflecting an energy

deficiency [14]. Continuous mitochondrial overload of fuel and incomplete fatty acid oxidation could lead to increased ROS production [106, 107]. Oxidative stress also impairs mitochondrial function, resulting in elevated ROS production and mitochondrial dysfunction. This mitochondrial dysfunction may in turn induce insulin resistance, resulting in a vicious cycle that leads to obesity-related pathogenesis. The elevated ROS level due to mitochondrial dysfunction stimulates various downstream pathways including JNK/signal transducers and activators of transcription (STAT) pathway, JNK/ inhibitor of nuclear factor kappa-B kinase (IKKβ), and MAPK, which can disturb leptin and insulin pathways [52, 108-110]. A decreased number of mitochondria may also lead to insulin resistance [52]. Petersen and colleagues demonstrated that insulin resistance is caused by dysregulation of cellular fatty acid metabolism, which is a result of an inherited defect in mitochondrial oxidative phosphorylation [111].

3.3. ER stress and obesity

ER, an important organelle that forms an interconnected network, is responsible for the synthesis, folding, and maturation of secreted and transmembrane proteins; biosynthesis of lipids; and the storage of Ca^{2+} [112, 113]. ER stress results from an imbalance between the protein folding capacity and the protein load [55]. The direct result of ER stress is the accumulation of misfolded protein. The ER establishes direct contact with mitochondria through ER domains termed mitochondrial-associated membranes (MAMs). MAMs allow bidirectional communication and trafficking of a signaling molecule [114]. Such association is essential for both mitochondria and ER functions. The interaction between the ER and mitochondria is partially modulated by mitochondrial fusion and fission [114]. The dysfunction of mitochondria is one trigger to ER stress through decreased ATP production and the alteration of the mitochondrial membrane potential and permeability. Both diet-induced and genetic obesity in mice are associated with increased ER stress in the hypothalamus [56, 115, 116]. Hypothalamic

ER stress has emerged as a causative factor in the development of obesity by inducing leptin resistance and insulin resistance.

Enhanced hypothalamic ER stress may play a primary pathogenic role in the development of leptin resistance. Schneeberger et al. put forward the hypothesis that MFN2 in the POMC is the molecular link between hypothalamic ER stress and leptin resistance [13]. To explore whether inhibition of ER stress could reverse the sensitivity of leptin, different approaches aimed at alleviating hypothalamic ER stress were conducted. Diet induced obese mice were treated with chaperones 4-phenylbutyrate (4-PBA) or tauroursodeoxycholic acid (TUDCA). The results showed normalization of the expression of ER stress, accompanied by enhanced leptin sensitivity and reduced food intake and body weight [56, 115-118]. Furthermore, ER stress inducing reagents could inhibit leptin signaling by blocking the phosphorylation of STAT3 [119]. Research also found that ER stress leads to leptin resistance via the mediation of protein tyrosine phosphatase 1B (PTP1B) [118].

Besides leptin resistance, ER stress may induce insulin resistance as well. The activation of JNK by ER stress could lead to increased serine phosphorylation of IRS. Phosphorylation of IRS at particular serine residues will disturb the interaction of IRS with InsR [120]. In addition, ER stress in pancreatic β-cells leads to β-cell apoptosis [121]. ER stress could also induce leptin/insulin resistance through elevated inflammation in the hypothalamus [55]. The mechanism of inflammation during the development of obesity will be discussed in the following section.

3.4. Hypothalamic inflammation and obesity

Continuous excessive nutrient exposure of the mitochondria could activate inflammatory pathways both in peripheral metabolic organs and in the CNS. Hypothalamic inflammation is characterized by increased interleukin and cytokine levels. For example, increased expression of the proinflammatory cytokines including IL-1, IL-6, and tumor necrosis factor alpha (TNFα), as

well as the elevated expression of IKKβ, an important upstream kinase regulator of nuclear factor kappa–B (NF-κβ), were observed in the hypothalamus of obese rats [122, 123].

Both lipid infusion and high-fat diet feeding could lead to obesity by activating hypothalamic inflammatory signaling pathways, which results in enhanced food intake and increased nutrient storage. Saturated fatty acids activate neuronal JNK and NF-κB signaling pathways, which directly impact leptin and insulin signaling [56]. The neuronal pro-inflammatory signaling could disturb intracellular signal transduction downstream of insulin receptors through the IRS phosphatidylinositol 3-kinase pathway [124]. Hypothalamic inflammation impairs leptin signaling via the JAK/STAT signaling pathway [125, 126] and induces obesity by up-regulating molecules and pathways including an inhibitor of IKKβ, serine kinases, JNK, toll-like receptor 4 (TLR4), the ceramide biosynthesis pathway, and/or the ER stress pathway [127, 128]. The induction of hypothalamic inflammation is associated with the activation of toll-like receptor (TLR) signaling [127]. Enhanced TLR expression activates JNK and IKK signaling and stimulates expression of inflammatory genes, such as TNFα and IL1β, which may be the main contributors to leptin resistance and insulin resistance. When TLR4 signaling was blocked, leptin resistance and insulin resistance were reversed [127].

4. Carotenoids, BCO2, and mitochondrial function

Carotenoids are a group of fat-soluble pigments derived from a 40-carbon base structure. Based on the chemical structure, carotenoids can be categorized into two classes: (1) carotenes, which contain only carbon and hydrogen atoms, and (2) oxocarotenoids (xanthophylls), which carry at least one oxygen atom [129].

Carotenoids are crucial for brain and visual function. Lutein and zeaxanthin are dominant carotenoids in human brain tissue, which account for 66–77% of total carotenoid concentration in

the brain [130, 131]. They are also believed to prevent damage that leads to age-related macular degeneration by quenching free radicals and absorbing blue light [132, 133]. Significant correlation between macular pigment density and global cognitive function was found in older adults [134, 135]. It was reported that macular lutein and zeaxanthin were significantly correlated with their levels in brain tissue [136]. Therefore, macular pigment can also be used as a biomarker of lutein in brain tissue [137]. The brain is vulnerable to free radical attack because of the high metabolic activity, and high polyunsaturated fatty acid content. Lutein and zeaxanthin are the predominant carotenoids that are selectively taken up by human brain tissue and play an important neuro-protective role in brain function [138]. The most well-known benefit of carotenoids in brain is improvement of cognitive function [137]. These carotenoids have also been suggested to modulate functional properties of synaptic membranes and to be involved in certain changes in the physiochemical and structural features of the membranes [139]. Most of these functions are attributed to their anti-oxidative and anti-inflammatory properties [137].

Considering the critical roles of carotenoids in brain function, a well-regulated metabolism of carotenoids is necessary for brain, as well as the whole body health. BCO2 is a mitochondrial enzyme involved in the cleavage of carotenoids. Compared to β-carotene-15, 15'-monooxygenase (BCO1), BCO2 exhibits broader substrate specificity. It catalyzes asymmetric cleavage of both provitamin A and non-provitamin A carotenoids at the 9', 10' double bond to form β-ionone and β-apo-10'-carotenal [15]. The metabolites of carotenoids also exhibit important biological function such as the activation of the NF-κB transcription system which is associated with harmful effect to bone health, and contributes to cancer development. The 10,10′-diapocarotene-10,10′-dial, which is the cleave products of lycopene by BCO2, was reported to inhibit NF-κB transcription system and to reduce the expression of NF-κB target genes [140]. Such process was mediated by the direct interaction of carotenoid derivatives with key kinases IKKβ and the p65 subunit. The fact that only the carotenoid derivatives, rather than the intact

carotenoid exhibited inhibitory effect on NF-κB highlights the critical role of BCO2 in providing the health benefits of carotenoids.

Previously, most research related to BCO2 was focused on the enzymatic function of BCO2. For example, Amengual et al. reported the deletion of BCO2 led to the disrupted carotenoid homeostasis accompanied by the elevated oxidative and reduced respiratory activity when mice were fed with diets rich in carotenoids [23]. However, our recent studies found that deletion of BCO2 gene in mice leads to increased appetite and higher risk of obesity, even when mice were fed with chow diet or high fat diet without carotenoids. This observation brings the possibility that BCO2 may play a more complicated role in maintaining mitochondrial function to modulate whole body energy homeostasis, rather than just exhibiting enzymatic function. The proteomics and metabolomics analysis from our laboratory further revealed perturbed energy metabolism, mitochondrial dysfunction, and elevated oxidative stress in hypothalamus caused by deletion of BCO2.

Conclusion and perspectives

The hypothalamus and mitochondria are two important centers for regulation of energy homeostasis at the organ and organelle levels, respectively. As the key organelle that maintains cellular energy metabolism, hypothalamic mitochondria participate in a variety of cell signaling pathways and modulate energy homeostasis through mitochondrial dynamics. Mitochondria is involved in the development of obesity via inadequate ATP production, enhanced inflammation, elevated oxidative stress, and ER stress. Therefore, the mitochondrial integrity is critical for energy homeostasis of the whole body and hypothalamus. Carotenoids have been recently characterized in brain. However, the functions of carotenoids and the metabolites in hypothalamus are poorly understood.

Considering the potential role of BCO2 in mitochondrial structure and function and the key position of the hypothalamus in energy balance, BCO2 may play a novel function in maintaining metabolic homeostasis in hypothalamus through regulation of mitochondrial dynamics. However, the exact mechanism by which BCO2 mediates mitochondrial dynamics and hypothalamic function in regulating energy balance remains unknown. Elucidating how BCO2 and the carotenoid metabolites protect mitochondrial activity and subsequent contribution to maintaining hypothalamic function will provide new perspectives for the prevention and treatment of obesity.

CHAPTER III

ALTERED LIPID METABOLISM REMODELS HYPOTHALAMIC METABOLOME TO STIMULATE FEEDING BEHAVIOR IN $BCO2^{-/-}$ MICE

1. Introduction

Excess food intake is a major contributor to the high incidence of obesity, which has emerged as a serious health issue around the world. Such feeding behavior requires a coordinated effort of neurotransmitters, peptides, and hormones in various nuclei throughout the brain [141]. The hypothalamus is a key brain region for modulating feeding behavior through signal integration from central and peripheral pathways. Two functionally distinct populations of neurons, which express POMC and AgRP/NPY, could respond to a variety of circulating hormones to regulate both short- and long-term energy homeostasis [38].

Glucose, amino acid, and fatty acid all function as signaling molecules which can be detected by the hypothalamus to modulate feeding behavior [9]. There are two types of glucosensing neurons that are either inhibited or excited as glucose level fluctuates [60]. Glucosensing neurons regulate their membrane potential, firing rate, and ion channel function to sense ambient glucose. Glucose sensing is especially important in stimulating appetite when

glucose availability drops [63]. Some amino acids, especially branched chain amino acids (BCAAs), may also produce signals relevant to food intake and protein balance [142]. The administration of leucine into mediobasal hypothalamus suppressed food intake [143]. The injection of BCAAs metabolites, such as α-ketoisocaproic acid, or α-ketoisovaleric acid into mediobasal hypothalamus lead to reduced food intake and body weight [143]. There is also increasing evidence indicating fatty acid sensing as important contributor in the regulation of feeding behavior, energy utilization, and storage [144]. Brain fatty acids act as a satiety signal to inhibit appetite [71]. Both the peripheral and central administration of fatty acid synthase inhibitor significantly suppressed appetite [72, 73]. *In vivo* study showed that food intake and hepatic glucose production was inhibited after three days intracerebroventricular infusion of oleic acid [74]. LCFA, such as oleic acid, might activate POMC neurons via the inhibition of K_{ATP} channel activity [145].

The carotenoids, carotenoid-derived products, and carotenoids-cleavage enzymes are reported to be involved in lipid metabolism, adipocyte biology, and fat accumulation [146]. Two enzymes are involved in the cleavage of carotenoids. β, β-carotene-15',15'-oxygenase1 (BCO1) catalyzes the conversion of β-carotene into retinaldehyde; and β, β-carotene-9',10'-oxygenase2 (BCO2) into β-10'-apocarotenal and β-ionone [147]. These compounds were reported to reduce adiposity by affecting adipocyte differentiation [148], hypertrophy [146], basal lipolysis, fatty acid oxidation [149], thermogenesis [150], and secretory function [146]. The administration of β-carotene to wild type mice significantly reduced adipose tissue weight and adipocyte size [147]. The expression of peroxisome proliferator-activated receptor γ (PPARγ) was found to be decreased at both mRNA and protein level [147] in wild type mice. However, these effects were not observed in BCO1 knockout mice, which indicated the critical role of carotene-cleaved products, rather than carotene itself in lipid metabolism. Furthermore, the reduced levels of

triacylglycerol, cholesteryl ester, phosphatidylinositol, and ceramide in the brain of BCO1 knockout embryos [151] also reveal the importance of BCO1 in lipid metabolism.

Unlike BCO1, which is a cytosolic protein, BCO2 was found to be in the inner mitochondrial membrane [31], where complexes of the ETC/OXPHOS are located. Carotenoid homeostasis was disrupted in $BCO2^{-/-}$ mice, accompanied by the accumulation of carotenoids in the mitochondria [23]. This led to mitochondrial dysfunction as indicated by increased MnSOD level, elevated oxidative stress, and decreased respiratory activity [23]. Our previous study also showed that $BCO2^{-/-}$ mice exhibited higher food intake and were more prone to obesity. In addition, BCO2 protein expression was decreased in obese and diabetic mice [20, 21]. The aim of this study is to explore the mechanism by which BCO2-regulated lipid metabolism impacts hypothalamic metabolome and subsequent stimulation of feeding behavior. Here, plasma biomarkers of lipid metabolism, metabolomics profiling were utilized to investigate how hypothalamic metabolome were altered by BCO2 deletion.

2. Methods

2.1. Animals and animal care

Male wildtype (WT) and $BCO2^{-/-}$ mice at six weeks of age were used in this study and group-housed (3 mice/cage) in a controlled environment with a 12-h/12-h light/dark cycle. Mice were fed a regular chow diet (CD, 13.4% kcal from fat, #5001; LabDiet, St Louis, MO). All mice had free access to water and food throughout the study. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University. Hypothalamic tissues were collected for laboratory analysis.

2.2. Plasma lipids analysis

Blood samples from WT and $BCO2^{-/-}$ mice were collected and centrifuged for 20 min at 2000 rpm at 4°C and stored at −80°C. The plasma non-esterified fatty acids (NEFA), triglyceride (TG), HDL, and LDL was quantitated using a Biolis 24i Analyzer (Carolina Liquid Chemistries, Winston-Salem, NC).

2.3. Western blot analysis

Protein samples prepared from hypothalami [21] were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and blocked in 5% nonfat milk for 1 hour followed by incubation in primary antibodies, including AKT, phospho-AKT, ACC, and β-actin (rabbit, dilution 1:1000, Cell signaling) overnight at 4 ºC. Membranes were washed with PBS followed by incubation with secondary antibody, anti-rabbit IgG, HRP-linked Antibody (rabbit, dilution 1:1000, Cell signaling). Immunoreactive bands were detected by chemiluminescence (SuperSignal Chemiluminescent Substrate; Thermo Fisher Scientific) and visualized using FluorChem R System (ProteinSimple, USA). Protein expression level was normalized using β-actin as loading control.

2.4. Global metabolomic profiling

To examine the functional impact of BCO2 on global metabolic phenotype in the hypothalamus of mice, the global metabolomic profiling was conducted by Metabolon, Inc (Durham, NC). The metabolites of hypothalamus samples from WT and $BCO2^{-/-}$ mice (6 technical replicates for each genotype) were identified by ultra-high performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) and gas chromatography-mass spectroscopy (GC-MS).

2.5. Ingenuity pathway analysis (IPA)

The metabolites identified in global metabolomic profiling with their p-value and fold change were uploaded to the IPA-Metabolomics platform [152]. This platform is capable of integrating proteomics and transcriptomics data to find significantly perturbed pathways, metabolic regulators, and novel biomarkers.

2.6. Quantitation of cytokines expression level

The Bio-Plex Mouse Diabetes 8-plex Panel assay kit (Bio-Plex Cytokine reagent kit, BIO-RAD Laboratories, Hercules, CA, USA) containing fluorescent microspheres conjugated with monoclonal antibodies were used to test the plasma insulin, glucagon, and leptin level between WT and $BCO2^{-/-}$ mice. Plasma samples were diluted four fold by adding 30μL serum and 120μL sample diluent. All procedures were performed following manufacturer's instructions. Samples were incubated with antibody-coupled beads, biotinylated secondary antibodies, and streptavidin-phycoerythrin. Bead fluorescence intensity was detected by Bio-Plex MAGPIX multiplex reader (Bio-Rad, USA), and data analysis was performed with the Bio-Plex-Manager software (BIORAD Laboratories, Hercules, Calif., USA).

2.7. Statistical analyses

Data were presented as mean ± SD. For Global metabolomic profiling, Welch's two-sample *t*-test, matched pairs t-test, one-way ANOVA, and two-way ANOVA were used. Hotelling's T2 test, random forest, and principal components analysis (PCA) were used for significance tests and classification analysis. The difference of plasma parameters level and protein expression level between genotypes were analyzed using student's *t*-test. An estimate of the false discovery rate (q-value) is calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Impact of BCO2 on plasma lipids, glucose and hormones

The plasma parameters of WT and $BCO2^{-/-}$ mice were listed in **Table 1.** Both the fasting glucose and LDL increased significantly in $BCO2^{-/-}$ mice. The cholesterol and HDL level were significantly lower due to the deletion of BCO2. The insulin level did not change between two genotypes. However, the glucagon level decreased by 50.3%; and the leptin level decreased by 74.1% in $BCO2^{-/-}$ mice compared to their age-matched WT mice.

3.2. Global biochemical profiling

Four hundred seventy seven metabolites from the hypothalamus of WT and $BOC2^{-/-}$ mice were identified and profiled. The detected metabolites including amino acid, lipid, carbohydrate, nucleotide xenobiotics, cofactors and vitamins (**Fig. 2**), covering 72 biochemical pathways. Among the 98 meatbolites that reach significant level between genotypes($p<0.05$), 34 were significantly increased, while 64 metabolites were significantly decreased in $BCO2^{-/-}$ mice.

3.3. Random forest analysis

Random forest classification was applied to assess the capacity to distinguish different genotype, as well as identify metabolite that is important to classify. The resulting models correctly classified the 12 hypothalamus samples from WT and $BCO2^{-/-}$ group with 100% predictive accuracy, which indicated genotypes displayed distinctive metabolic phenotypes. Mean decrease accuracy, which indicates how much a certain metabolite contributes to separation of the two test groups, was computed for each biochemicals. Significantly changed metabolites with the mean decrease accuracy above 10 were listed in **Table 2**. Metabolic signatures indicating changes in LCFA oxidation (oleoylcarnitine, stearoylcarnitine, oleate), bile acid synthesis and liver function (tauro-β-muricholate, cholate, taurocholate, deoxycholate and methyl glucopyranoside), indicators of oxidative stress (methionine, S-methylcysteine, taurine, ophthalmate, 4-hydroxy-nonenal-glutathione, glutamine, betaine, kynurenine) and inflammation (histidine, 1-

methylimidazoleacetate, kynurenine, stearoyl-arachidonoyl-glycerophosphoinositol, stearoyl-oleoyl-glycerophosphoserine) were among the key factors prominent in genotype separation.

Table 1 Altered plasma NEFA, glucose and hormones between WT and BCO2 $^{-/-}$ mice

Parameters	WT	BCO2-$^{-/-}$	P-value
Fasting blood glucose (mg/dL)	95.4 ± 16.2	135.3 ± 10.5	0.001
NEFA (mEq/L)	0.7 ± 0.1	0.96 ± 0.03	0.012
LDL (mg/dL)	6.6 ± 0.9	10.3 ±0.6	0.001
Cholesterol (mg/dL)	156.6 ± 18.5	109.5 ±4.0	0.002
HDL (mg/dL)	93 ± 8.4	55.5 ±3.1	0.000
Leptin (pg/mL)	2076.5 ± 225.0	537.8 ± 218.3	0.000
Glucagon (pg/mL)	63.4 ± 24.5	31.5 ± 2.1	0.041
Insulin (pg/ml)	29.3 ± 11.2	21 ±1.2	0.180

Values represent as mean ± SD

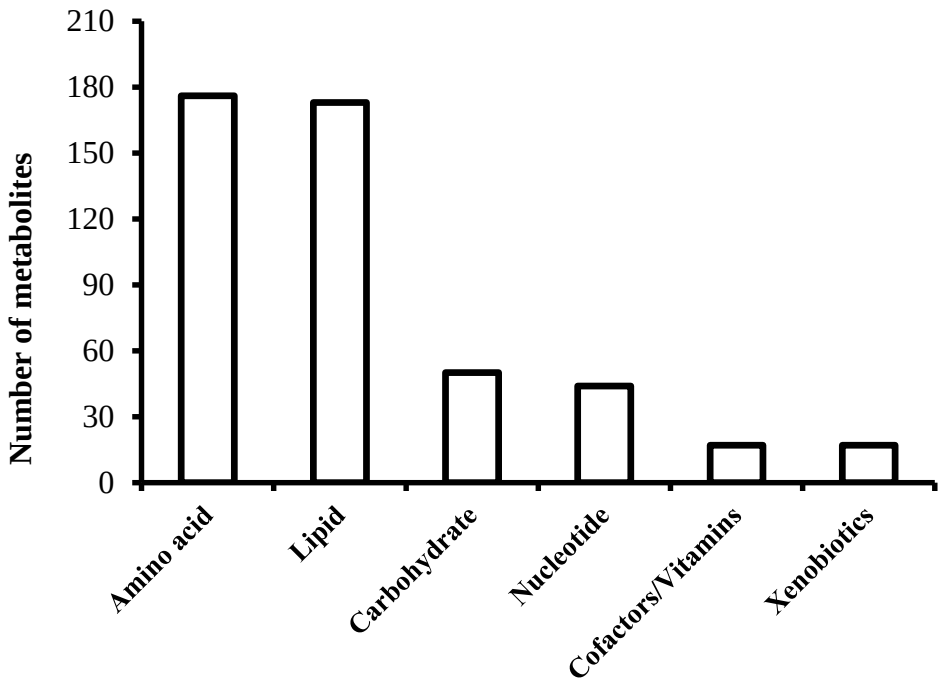


Fig. 2 Classification of metabolites detected in hypothalamus. Total number of metabolites based on biochemical classes. n=6.

Table 2 Biochemical importance table between WT and BCO2$^{-/-}$ mice

Biochemical Name	Mean decrease accuracy	Ratio (BCO2$^{-/-}$/WT)	Biochemical Name	Mean decrease accuracy	Ratio (BCO2$^{-/-}$/WT)
Amio acid metabolism			α-hydroxyisocaproate	10.8234	2.24 *
methionine	20.6643	0.78 ***	5-hydroxyindoleacetate	10.7699	0.62 **
indolelactate	20.173	0.23 ***	prolylvaline	10.4117	0.75 **
α-hydroxyisovalerate	20.1712	5.6 ***			
tyrosine	19.9737	0.63 *	**Lipidmetabolism**		
pipecolate	19.9522	1.83 ***	cholate	19.678	0.03 ***
urea	19.7835	1.42 ***	deoxycholate	19.5741	0.06 **
kynurenine	19.7163	0.42 ***	taurocholate	19.1587	0.09 **
isobutyrylcarnitine	19.5006	0.54 **	stearoylcarnitine	18.7763	0.7 **
γ-glutamyltryptophan	19.4767	0.34 ***	oleoylcarnitine	18.3828	0.72 **
tryptophan	18.9214	0.59 ***	N-palmitoyltaurine	18.2983	2.04 *
prolylalanine	18.8363	0.86 **	tauro-β-muricholate	15.6101	0.19 *
histidine	18.7456	0.69 **	N-oleoyltaurine	14.4865	1.59 ***
glycylvaline	18.6505	0.65 **	1-palmitoylglycerophosphoserine	14.3495	3.5 **
allo-threonine	18.6337	2.47 *	1-stearoylglycerophosphoserine	12.6341	1.43 *
3-(4-hydroxyphenyl)lactate	18.578	0.38 *	N-stearoyltaurine	12.0064	1.82 **
phenylalanine	18.5146	0.7 *	oleate (18:1 n9)	11.7209	0.81 *
3-indoxyl sulfate	18.5091	0.17 ***	stearoyl-arachidonoyl-glycerophosphoinositol	11.4019	0.71 *
N-acetyltyrosine	18.4288	0.42 *	stearoyl-oleoyl-glycerophosphoserine	11.1279	2.12 *
3-methylglutarylcarnitine	18.3195	1.81 **			
γ-glutamylglutamine	17.928	0.65 *	**Carbohydrate metabolism**		
ophthalmate	17.7367	1.67 ***	sucrose	20.1652	26.84 **
prolylglutamine	17.7356	0.34 *	1,5-anhydroglucitol	18.9579	1.99 ***
S-methylcysteine	17.5736	0.24 **			
1-methylimidazoleacetate	14.9067	0.54 **	**Nucleotide metabolism**		
γ-glutamylphenylalanine	14.8001	0.56 *	thymidine	19.7096	0.66 **
C-glycosyltryptophan	14.7692	1.21 **	adenine	18.3837	0.82 ***
betaine	14.302	0.69 *	2'-deoxyuridine	13.5304	0.69 **
γ-glutamyltyrosine	14.0757	0.48 **			
alanylalanine	13.6308	1.55 **	**Cofactors and Vitamins**		
N-acetylglutamine	13.5631	0.41 *	pantothenate	19.8491	0.5 ***
2-hydroxy-3-methylvalerate	13.5603	3.01 *	riboflavin (Vitamin B2)	16.0771	0.76 *
4-hydroxy-nonenal-glutathione	13.4777	1.36 **	pyridoxal	11.3895	0.8 *
N-acetylphenylalanine	13.4647	0.28 *			
leucylglycine	13.0612	0.7 *	**Xenobiotics**		
N-acetyltaurine	12.7174	1.73 **	ergothioneine	19.0424	0.52 *
glutamine	11.9453	0.68 **	hippurate	18.2248	1.83 **
taurine	11.6979	1.32 **	methyl glucopyranoside	18.0238	1.56 ***
glycylleucine	10.845	0.83 *	catechol sulfate	11.7362	0.63 *

* indicate p-value<0.05; ** indicate p-value<0.01; *** indicate p-value<0.001.

3.4. Altered fatty acid metabolism

LCFAs, such as oleate (18:1 n9) (**Fig. 3A)**, and docosahexaenoate (DHA; 22:6n3) (**Fig. 3B)**, were significantly lower in $BCO2^{-/-}$ mice. Long-chain acyl-carnitines and N-acyl-taurines in hypothalamus changed in reciprocal fashion. Long-chain acyl-carnitines, such as palmitoylcarnitine (**Fig. 3C**), stearoylcarnitine (**Fig. 3D)**, and oleoylcarnitine (**Fig. 3E)** were significantly decreased in $BCO2^{-/-}$ hypothalamus with respect to WT, whereas N-acyl-taurines such as, N -palmitoyltaurine (**Fig. 3F)**, N-stearoyltaurine (**Fig. 3G)**, and N-oleoyltaurine (**Fig. 3H**) were elevated. Acyl-carnitine conjugation by carnitine palmitoyltransferase (CPT) is required for import of LCFA into the mitochondria before fatty acid oxidation. Lower long-chain acyl-carnitines level in the hypothalamus indicated decreased transport of LCFAs into the mitochondria.

To determine whether or not $BCO2^{-/-}$ impacts fatty acid synthesis, the expression level of acetyl-CoA carboxylase (ACC), which catalyze the rate-limiting step of fatty acid biosynthesis was tested in WT and $BCO2^{-/-}$ mice. Result showed that ACC level tended to be higher in $BCO2^{-/-}$ mice ($0.05<p<0.1$) **(Fig. 3 I-J)**.

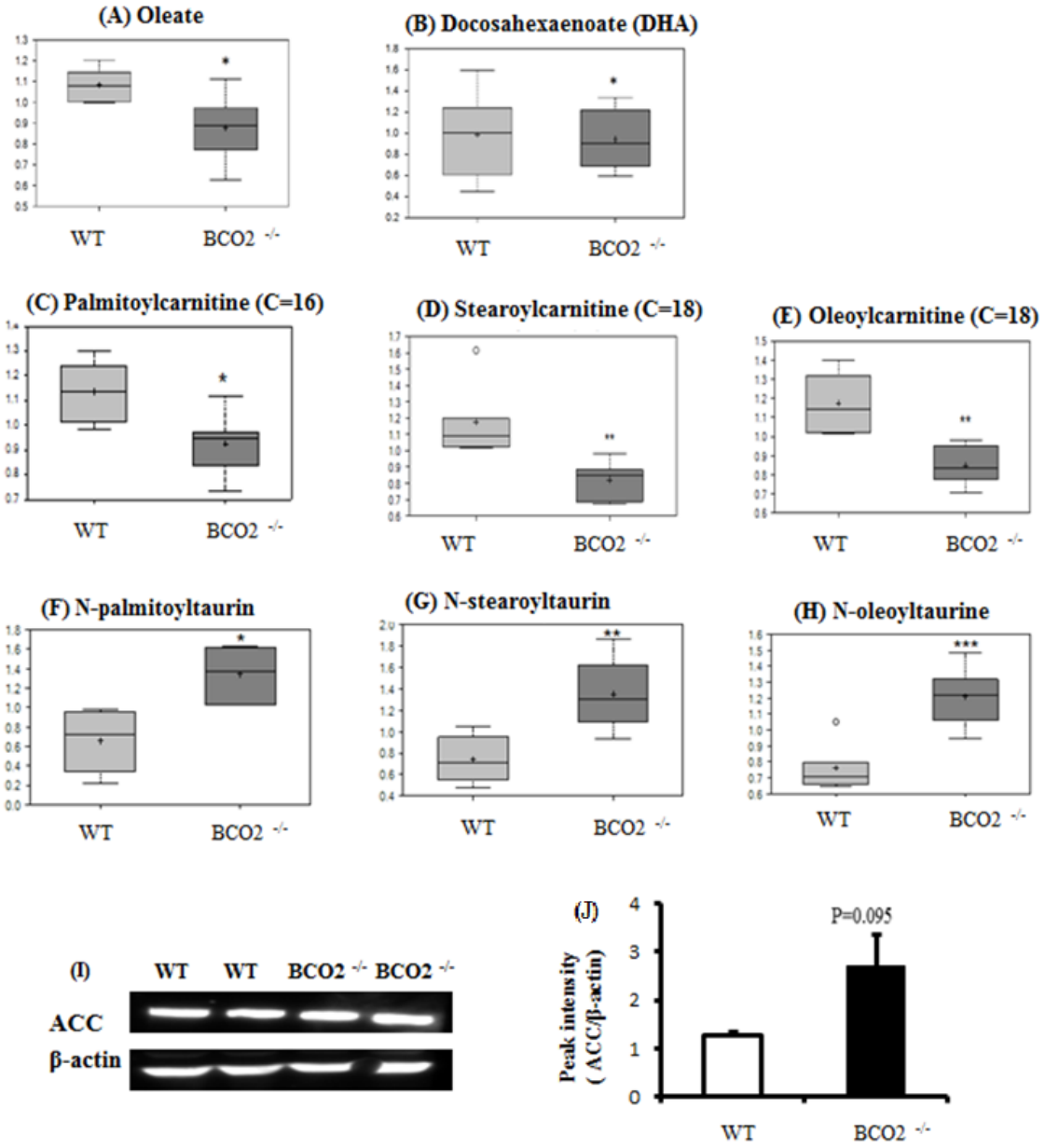


Fig. 3 The impact of BCO2 on lipids metabolism. Effect of BCO2 on **(A)** oleate**, (B)** docosahexaenoate**, (C)** palmitoylcarnitine, **(D)** stearoylcarnitine, **(E)** oleoylcarnitine, **(F)** N-palmitoyltaurine**, (G)** N-stearoyltaurine**, (H)** N-oleoyltaurine in the hypothalamus of $BCO2^{-/-}$ mice compared to WT mice. The tops and bottoms of the boxes represent the interquartile range, the "+" represents mean value, the solid line in the middle represents the median, and the bars (whiskers) representing the range of the data points, the "o" represents extreme data point, n = 6. **(I)** The expression level of ACC and **(J)** quantification of ACC between $BCO2^{-/-}$ mice and WT mice. n = 2. ACC, acetyl-CoA carboxylase WT, wild type. *0.01<P<0.05; **0.001<P<0.01; *** P<0.001.

3.5. The change in monosaccharide metabolism

Similar to the decreased LCFA level in hypothalamus of $BCO2^{-/-}$ mice, the glucose level was also significantly lower in $BCO2^{-/-}$ mice (**Fig. 4A**). Contrary to the decreased transport of LCFA for β-oxidation, transport of D-glucose and other monosaccharides was activated in the $BCO2^{-/-}$ group (**Fig. 4B)** as predicted by the IPA system**.** The expression of protein kinase B (PKB), also known as AKT, which is a protein kinase that plays a key role in insulin signaling pathway and glucose uptake, was not changed (**Fig. 4C-D**). However, the phosphorylated AKT was inhibited in $BCO2^{-/-}$ mice (**Fig. 4E**).

3.6. Disrupted sterol metabolism

Markers associated with sterol metabolism and the conversion of cholesterol to primary and secondary bile acids were disrupted in $BCO2^{-/-}$ mice **(Fig. 5A)**. The cholesterol level was significantly decreased in $BCO2^{-/-}$ mice **(Fig. 5B)**. This may be due to the elevated 3-hydroxy-3-methylglutarate (HMG) activity **(Fig. 5C).** Increased HMG suggests the rate-limiting step of cholesterol synthesis-the conversion of HMG-CoA to mevalonate-was restricted in knockout animals. This led to the alternative disposal of HMG-CoA via the formation of HMG.

Bile acid biosynthesis was inhibited in $BCO2^{-/-}$ mice. Decreased levels of cholate **(Fig. 5D)**, and taurine-conjugate tauroursodeoxycholate **(Fig. 5E)** indicated that the classic bile acid synthesis pathway was inhibited in $BCO2^{-/-}$ mice. The decreased hydroxy-3-oxo-4-cholestenoate (7-HOCA) **(Fig. 5F)** reflected the alternative pathway was also inhibited. Furthermore, the secondary bile acids deoxycholate was also decreased in $BCO2^{-/-}$ mice **(Fig. 5G)**. Therefore, both primary and secondary bile acids and their conjugates were decreased in $BCO2^{-/-}$ mice as compared to WT mice.

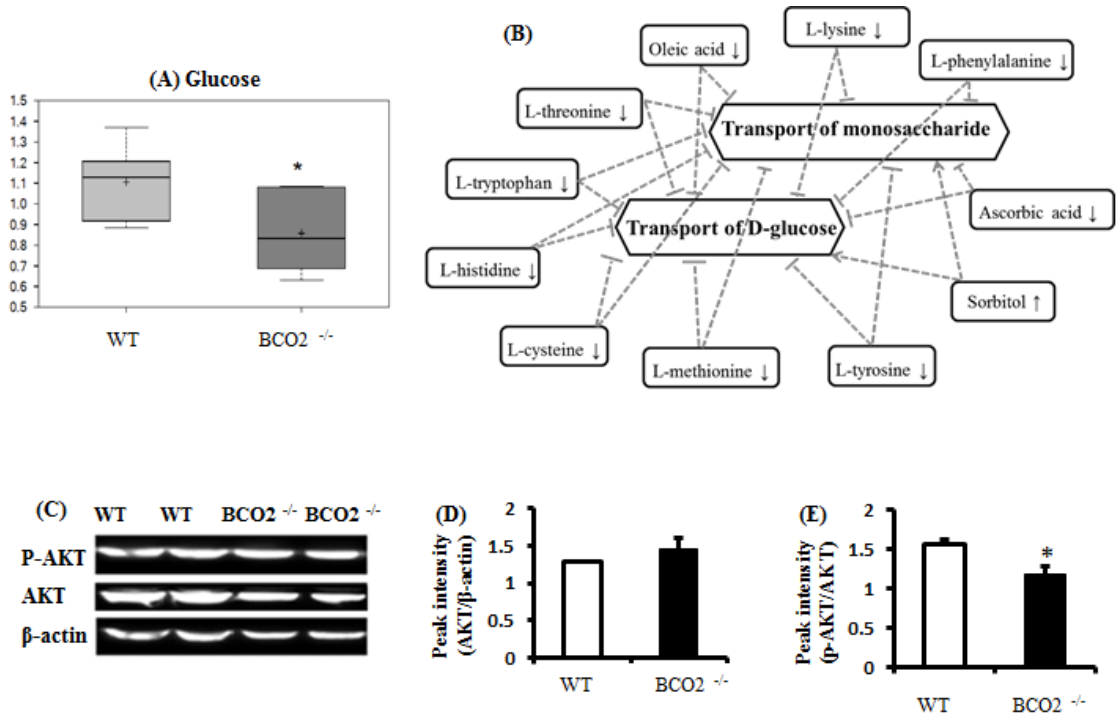


Fig. 4 The impact of BCO2 on glucose metabolism. (A) Decreased glucose level in hypothalamus. The tops and bottoms of the boxes represent the interquartile range, the "+" represents mean value, the solid line in the middle represents the median, and the bars (whiskers) representing the range of the data points, the "o" represents extreme data point, $n = 6$. **(B)** Increased transport of monosaccharides in $BCO2^{-/-}$ mice predicted by Ingenuity Pathway Analysis. **(C)** The change of AKT expression and **(D-E)** quantification of AKT between $BCO2^{-/-}$ mice and WT mice. $n = 2$. AKT, Protein kinase B; P-AKT, phosphorylated AKT; WT, wild type. $*0.01<P<0.05$.

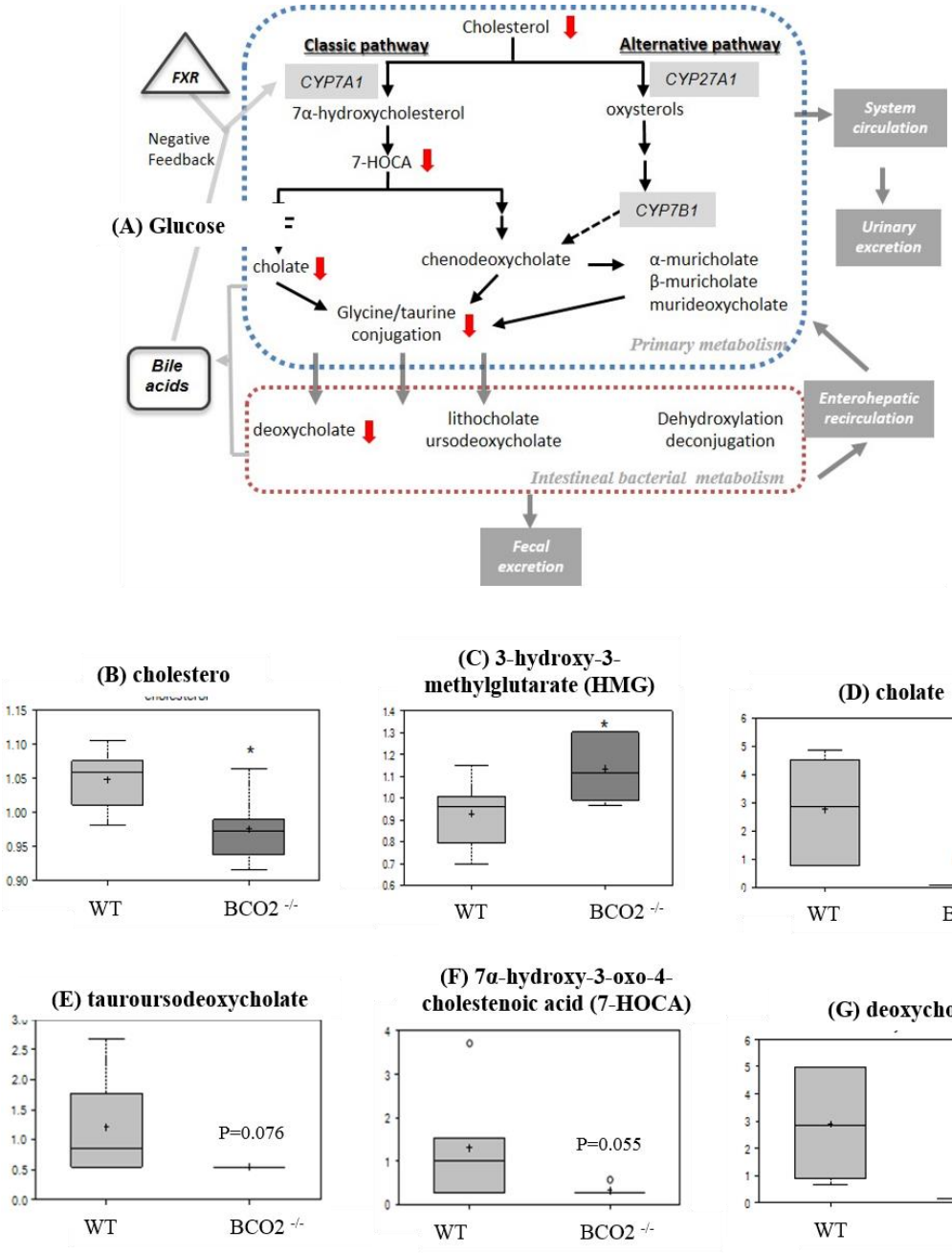


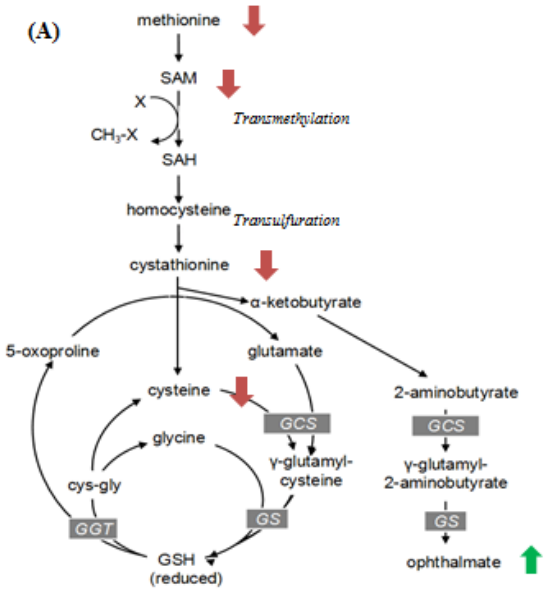
Fig. 5 Decreased bile acid metabolism in BCO2 $^{-/-}$ mice compared to WT mice. (A) Classic" and "Alternative" pathway of bile acid synthesis**.** The change of cholesterol **(B),** 3-hydroxy-3-methylglutarate (HMG) **(C)**, cholate **(D)**, tauroursodeoxycholate **(E)**, 7α-hydroxy-3-oxo-4-cholestenoic acid (7-HOCA) **(F)**, and deoxycholate **(G)** in hypothalamus of BCO2$^{-/-}$ mice compared to WT mice. The tops and bottoms of the boxes represent the interquartile range, the "+" represents mean value, the solid line in the middle represents the median, and the bars (whiskers) representing the range of the data points, the "o" represents extreme data point, n = 6. FXR, farnesoid X receptor; 7-HOCA, 7-α-hydroxy-3-oxo-4-cholestenoate. WT, wild type. *0.01<P<0.05; **0.001<P<0.01; *** P<0.001.

3.7. Elevated oxidative stress and inflammation in BCO2$^{-/-}$ mice

The metabolism of sulfur-containing amino acids is important for one carbon transfer catalyzed by methyltransferases as well as for converting methionine into antioxidants such as cysteine and glutathione sulfhydryl-containing derivatives (**Fig. 6A**). The results from global metabolon revealed methionine and glutathione transsulfuration was inhibited in BCO2$^{-/-}$ mice. A significant decrease in sulfur-containing amino acids such as methionine, S-adenosylmethionine(SAM**)**, cystathionine and cysteine was observed in BCO2$^{-/-}$ mice compared to wild-type mice (**Fig. 6B**). The elevated level of ophthalmate (OPH), combined with the decreased cystathionine and cysteine level in BCO2$^{-/-}$ mice, indicated that cystathionine may predominantly convert to α-ketobutyrate and finally form ophthalmate rather than convert to cysteine. Significantly increased 4-hydroxy-nonenal-glutathione (4-HNE-GS) and increased trend of erythronate (P=0.075), both of which are oxidative stress biomarkers, were also observed in BCO2$^{-/-}$ mice.

Markers of histamine metabolism 1-methylimidazoleacetate was lower in hypothalamus, which suggests the basophils - major producers of histamine - may have been relatively less active in the hypothalamus of BCO2$^{-/-}$ mice. Decreased histidine (**Fig. 6B**) and oleate **(Fig. 8A)**, which exhibit anti-inflammatory effects, were observed in BCO2$^{-/-}$ mice.

The glycerophosphoinositol- and glycerophosphoserine- containing fatty acyl groups also changed between WT and BCO2$^{-/-}$ mice. The glycerophosphoinositols play a role in inflammatory and immune responses and function as modulators of T-cell signaling and T-cell responses [153]. Glycerophosphoserines are also involved in cell signaling, especially during macrophage recognition of cells that undergo apoptosis [154].



(B)

LOW HIGH

WT						BCO2 -/-						Biochemical Name	Sub Pathway
1	2	3	4	5	6	1	2	3	4	5	6		
												methionine	
												S-adenosylmethionine (SAM)	Methionine, Cysteine, SAM and Taurine Metabolism
												cystathionine	
												cysteine	
												ophthalmate (OPH)	Glutathione Metabolism
												4-hydroxy-nonenal-glutathione (4-HNE-GS)	
												1-methylimidazoleacetate	Lysine Metabolism
												histidine	Histidine Metabolism

Fig. 6 Elevated oxidative stress and inflammation in hypothalamus. (A) Methionine/Glutathione transsulfuration pathway; **(B)**The change of metabolites and the pathway related to sulfur-containing amino acids and lysolipids. Heat map depicting significant change in sulfur-containing amino acids and lysolipids after the deletion of BCO2, as analyzed by LC-MS/MS . Red indicates lower level; while green indicates higher level; WT, wild type. n=6 .

4. Discussion

4.1. Altered hormone and nutrients sensing in hypothalamus contribute to feeding behavior change in BCO2$^{-/-}$ mice

Our previous data showed BCO2$^{-/-}$ mice always exhibit higher food intake compared to age matched mice. The significant lower circulating leptin levels observed in BCO2$^{-/-}$ mice functions as a contributory factor in enhanced appetite. Leptin is an anorectic hormone that binds to the leptin receptor (LepR) in hypothalamus to initiate downstream signaling through the phosphorylation of transcription factor signal transducer and activators of transcription (STAT) and tyrosine kinase Janus kinase (JNK) [155]. In the STAT family, STAT3 is mostly involved in leptin-mediated anorectic effects [156]. Phosphorylation of STAT3 participates in the leptin-induced transcriptional regulation of appetite regulation neuropeptides, such as POMC, AgRP, and NPY.

In addition to lower circulating leptin in BCO2$^{-/-}$ mice, hypothalamic nutrient sensing is also involved in regulatingfeeding behavior. The metabolic-sensing neurons in hypothalamus regulate peripheral fuel availability by altering behavior in response to glucose and fatty acids levels [157]. BCO2$^{-/-}$ mice exhibited significant lower glucose in hypothalamus. Glucose sensing is especially important in stimulating appetite when glucose availability drops. Hypothalamic glucose level modulates ingestive behavior by regulating GLUT2, glucokinase, and K_{ATP} channels [63]. Lower glucose in hypothalamus may reflect an energy deficiency status in BCO2$^{-/-}$ mice, thus the BCO2$^{-/-}$ mice exhibit orexigenic phenotype.

Oleate was significantly decreased in BCO2$^{-/-}$ mice. Approximately 50% of metabolic-sensing neurons responded to oleic acid by using the FA translocator/receptor FAT/CD36 (CD36) [158]. Oleic acid may acutely enhance hepatic insulin action via the activation of K_{ATP} channels in hypothalamus [74]. Oleic acid provides a signal of “nutrient abundance” in the CNS. This

signal in turn activates a series of neuronal events to promote a switch in fuel sources from carbohydrates to lipids [74]. Therefore, the decreased oleate level also indicated the tendency toward using carbohydrates as fuel in $BCO2^{-/-}$ mice.

The endocannabinoids, including N-oleoyltaurine, N-stearoyltaurine, and N-palmitoyltaurine, were significantly elevated in $BCO2^{-/-}$ mice. N-acyl taurines are relatively poorly understood members of the endocannabinoid family that are abundant in the brain, especially in CNS [159]. These N-acyl taurines may be synthesized from dietary n-3 and n-6 poly- unsaturated fatty acids by taurine-conjugating enzymes in the hypothalamus, or synthesized by acyl-coenzyme A:amino acid N-acyltransferase (ACNAT1) from peripheral tissue and transported to the CNS, or both [160]. The endocannabinoid system has emerged as a lipid signaling system in macronutrient metabolism in which the gastrointestinal system, liver, muscle, and adipose are all involved [161]. The endocannabinoids act as neurotransmitters between neurons in various regions of the brain, which may affect neuroendocrine functioning [162]. One physiological effect of the endocannabinoid system is to shift the energy balance toward energy storage and favor fat accumulation [163]. Increased endocannabinoids and activated cannabinoid receptors in the brain lead to increased appetite and obesity [161]. Furthermore, the N-taurine-conjugated fatty acids have been shown to activate transient receptor potential vanilloid 1 (TRPV1), and further trigger the release of free calcium ions to the cytosol, which stimulates insulin secretion from pancreatic β-cells [164].

4.2. Perturbed energy metabolism in $BCO2^{-/-}$ mice

Traditionally, only glucose was believed to cross the blood-brain barrier to provide ATP for the brain. Later, it was discovered that fatty acids are used by brain as well [165]. It has been reported that 20% of total energy expenses of the brain are satisfied by oxidation of fatty acids [166]. Free fatty acids need to be activated in the cytosol by forming fatty acyl-Coenzyme A

(CoA) before they get into mitochondria for β-oxidation. The carnitine palmitoyltransferase 1 (CPTI) on the outer mitochondrial membrane exchanges carnitine for the CoA group on LCFA to form a fatty acid-carnitine conjugate. Uptake of fatty acids into mitochondria is considered as the rate-limiting step of β-oxidation. The decrease of long-chain acyl-carnitines (palmitoylcarnitine, stearoylcarnitine, and oleoylcarnitine) in this case is consistent with decreased transport of LCFA into mitochondria.

The transport of monosaccharides also changed in $BCO2^{-/-}$ mice. The IPA software predicted the activation of the transport of D-glucose and other monosaccharides based on the increased sorbitol level; as well as decreased oleic acid, ascorbic acid, tyrosine, methionine, cysteine, histidine, tryptophan, threonine, lysine, and phenylalanine level. Sorbitol was reported to increase the transport of 2-deoxyglucose in C2C12 cells [167]. The administration of oleic acid in the intracerebroventricular of brain markedly inhibits endogenous glucose production. Ascorbic acid decreases transport of deoxyglucose in primary cultures of cortical and hippocampal neurons and in HEK293 cells [168]. Lysine, histidine, cysteine, tyrosine, tryptophan, threonine, phenylalanine, and methionine infusion are found to decrease the transport of D-glucose via inhibiting insulin receptor substrate-1 phosphorylation and decreasing insulin-induced phosphoinositide 3-kinase activity [169]. Therefore, the metabolites discussed above indicated that the transport of D-glucose and other monosaccharides might be upregulated due to the ablation of BCO2. In addition, the inhibition of AKT in $BCO2^{-/-}$ mice indicated inhibition of glucose uptake. AKT may activate glycogen synthesis through phosphorylation and inactivation of glycogen synthase kinase 3 (GSK-3) [170]. Overall, the results discussed above reveal that the depletion of BCO2 causes down regulation of LCFA oxidation, and leads to the tendency toward using glucose as substrate.

4.3. Decreased bile acid synthesis in $BCO2^{-/-}$ mice

Decreased intermediate products of bile acid synthesis indicated lower bile acid biosynthesis in $BCO2^{-/-}$ mice. Although the majority of bile acids are produced by liver, they can also be synthesized extrahepatically. Bile acid can either be synthesized or delivered to the brain [171, 172]. The pathway initiated by ER localized enzyme cholesterol 7α-hydroxylase (CYP7A1) is referred to as the classic (neutral) pathway of bile acid synthesis. The alternative (acidic) pathway of bile acid synthesis refers to the process that starts with the hydroxylation of cholesterol at the 27 position by the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1). For rodents, the acidic pathway can account for up to 25% of total bile acid synthesis [173]. Bile acids are synthesized extrahepatically via the acidic pathway starting with 27-hydroxycholesterol, or with 24S-hydroxycholesterol pathway [171, 174]. Both the initial steps of these two pathways occur in the brain [174]. Moreover, all enzymes needed for the biosynthesis of the primary bile acid by the 24S-hydroxycholesterol pathway are found to be expressed in the brain [171].

The sterols and bile acids may activate and act as ligands to many nuclear receptors in the brain, such as liver X receptors (LXRs) and Nur-related factor 1 (NURR1) [171]. The farnesoid X receptor (FXR) could also activated by bile acids, such as deoxycholic acid [175]. Therefore, the down-regulated bile acid synthesis and decreased cholate, tauroursodeoxycholate, 7-HOCA, and deoxycholate indicated less activation of these nuclear receptors.

The tauroursodeoxycholate in the brain was found to exhibit a protective role against motor and cognitive deficits and to reduce striatal degeneration during the development of Huntington's disease [172]. It has been reported that hydrophilic bile acids may exhibit antioxidant properties and interact with the mitochondrial membrane to prevent mitochondrial permeability transition (MPT), mitochondrial membrane depolarization, and cytochrome c release [172, 176]. Thus, the decreased bile acids level in $BCO2^{-/-}$ mice also indicates the risk of neuron and mitochondrial dysfunction.

4.4. Ablation of BCO2 leads to oxidative stress

Global metabolon data indicated decreased methionine, SAM, cystathionine and cysteine levels, as well as increased 4-HNE-GS and ophthalmate level. Methionine plays an important role against peroxidation of lipids, a process in which free radicals steal electrons from lipids in cell membranes, causing cell damage [177]. SAM is the major methyl donor in the body, and a provider of cysteine for synthesis of GSH. It may also function as a direct antioxidant in the cell [178]. Our results indicated reduced antioxidant activity in the hypothalamus after the knockout of BCO2. OPH is synthesized when GSH is depleted and when the hepatic availability of cysteine is limited. Although glutathione levels did not differ between hypothalamic samples taken from WT and $BCO2^{-/-}$ animals, the OPH, which is a marker of glutathione depletion, was significantly elevated in the $BCO2^{-/-}$ samples.

Increased 4-HNE-GS, a peroxidative stress biomarker, indicated that peroxidative stress was greater when BCO2 expression was suppressed and that glutathione was needed to neutralize these reactive compounds. Another elevated oxidative damage biomarker-erythronate, which is the oxidation product of N-acetylglucosamine, indicated that the loss of BCO2 expression in the hypothalamus led to an increased level of oxidative stress and damage [179]. Furthermore, HMG was reported to induce protein oxidative damage in cortical supernatants and to reduce nonenzymatic antioxidant potential and total antioxidant capacity in rat cerebral cortex [180]. Significantly higher HMG level also indicated elevated oxidative stress in $BCO2^{-/-}$ mice.

4.5. Increased risk of diabetes

AKT is an important signaling molecule in the insulin signaling pathway, which functions as an effector of the PI3K [181]. The major effect of AKT in glucose metabolism is the stimulation of glucose uptake in skeletal muscle, liver, adipocytes and other tissues by translocating GLUT4 from the intracellular pool to plasma membrane; and by promoting

glycogen synthesis [182]. Inhibition of AKT in $BCO2^{-/-}$ mice indicate decreased glucose uptake in peripheral tissues. The inhibited AKT may be associated with impaired insulin signaling pathways.

Significantly decreased glutamine, kynurenine, and tryptophan, and increased taurine were also associated with a high risk for developing diabetes in $BCO2^{-/-}$ mice. Metabolome analysis of spontaneously diabetic Torii (SDT) rat models identified significantly altered metabolites including glutamine, kynurenine, tryptophan, and taurine, which are all potential biomarkers for prediabetes [183]. Decreased tryptophan levels were observed in the hypothalamus [184] and other brain regions [185] in diabetic rats. L-glutamine was also found to promote insulin response, trigger GLP-1 release, and decrease postprandial glycaemia in type 2 diabetes patients [186]. A novel genetic locus located in the region of the GLUL gene on chromosome 1q25 was found to be associated with higher coronary heart disease risk among type 2 diabetic patients as a result of reduced GLUL gene expression [187].

4.6. Impaired brain and neuron function

Decreased betaine, 5-methylthioadenosine (MTA), and DHA are associated with impaired brain and neuron function. Betaine may delay the progression of Alzheimer's disease due to a decrease in serum homocysteine, and an increase of brain methionone and SAM [188]. Betaine also protects against rotenone-induced neurotoxicity in PC12 cells [189]. MTA displays a wide array of neuroprotective activities against different insults [190]. DHA is an omega-3 fatty acid, which is a critical structural component of the brain and important for central nervous system function [191]. DHA exhibits neuroprotective function, increases cerebral acetylcholine levels and enhances learning ability in rats [192]. It exihibits anti-inflammation role by inhibiting IL-1, IL-2, and TNF-α production [152]. It has also been proposed that neuron damage in the

ventromedial hypothalamus, which leads to the defects in insulin action, is a possible factor in the development of Type 2 diabetes [193].

4.7. BCO2 may be important for cellular metabolism and feeding behavior, rather than simply functioning as a carotenoids cleavage enzyme

It has been reported that carotenoids were found to accumulate in the mitochondria of $BCO2^{-/-}$ mice when mice were fed zeaxanthin or lutein diets [23]. The accumulated carotenoids led to mitochondrial dysfunction, as indicated by the increased MnSOD level and decreased respiratory activity [23]. However, in our study, no carotenoid was added into the diet. Therefore, BCO2 may maintain mitochondrial function not only through the homeostasis of carotenoid metabolism, but also through other mechanisms This study demonstrates that BCO2 depletion impacts energy metabolism, nutrient sensing, oxidative stress, and inflammation in mice at the level of the hypothalamus. The altered circulating hormone level, glucose and fatty acids sensing may contribute to the change of feeding behavior, thus resulting to the $BCO2^{-/-}$ mice exhibiting higher risk to develop obesity.

CHAPTER IV

THE ROLE OF BCO2 IN MAINTAINING HYPOTHALAMIC MITOCHONDRIAL PROTEOMICS AND RESPIRATORY ACTIVITY

1. Introduction

The hypothalamus is a key brain region for modulating feeding behavior and energy expenditure through nutrient sensing [8, 9] and signal integration from central and peripheral pathways [10]. The proopiomelanocortin (POMC) and agouti-related protein (AgRP)/neuropeptide Y (NPY) neurons response to leptin, insulin, and gut hormones, such as ghrelin, glucagon-like peptide-1, peptide YY3–36, cholecystokinin, and pancreatic polypeptide to maintain energy balance [10, 11]. Aside from the signals from hormones, the hypothalamus is also sensitive to circulating metabolites. Circulating lipids such as oleic acid provides a signal of "nutrient abundance" in the CNS. This signal in turn activates a series of neuronal events to promote a switch in fuel sources from carbohydrates to lipids [74]. Glucosensing neurons sense ambient glucose by regulating membrane potential, firing rates, and ion channels [60]. Glucose sensing is especially important in stimulating appetite when glucose availability drops [63].

Hypothalamic mitochondrial dynamics is also involved in the modulation of whole energy homeostasis [12, 13]. Mitochondria regulate AGRP and POMC neuronal activity through fission and fusion behavior and morphology changes in response to the environment especially to the energy status [85]. Furthermore, a possible source of leptin resistance may be attributed to

impaired mitochondrial ATP production. Research has shown that altered mitochondrial energy production, especially in skeletal muscles, is a major factor that disrups a chain of metabolic events leading to obesity [14]. Besides its function in energy production, mitochondria participate in many other processes including cell signalling, cell differentiation, and apoptosis [87]. Mitochondria are also involved in the generation of reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, and inflammation [194, 195]. Mitochondrial dysfunction is thought to trigger many chronic diseases, including obesity, diabetes, and neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease [97].

Recently, BCO2 (β,β-carotene-9',10'-oxygenase2) was reported to play an important role in protecting mitochondrial function [23]. BCO2 is an enzyme in the mitochondria that catalyzes asymmetric cleavage of both provitamin and non-provitamin A carotenoids at 9', 10' double bond to form β-ionone and β-apo-10'-carotenal [15]. BCO2 was found to be in the inner mitochondrial membrane, where complexes of the electron transport chain (ETC)/oxidative phosphorylation (OXPHOS) are located [31]. The deletion of BCO2 led to the disrupted carotenoid homeostasis accompanied by elevated oxidative and reduced respiratory activity when mice were fed with carotenoids [23].

However, our earlier finding showed $BCO2^{-/-}$ mice are more prone to obesity and exhibit higher food intake compare to wide type (WT) mice, even when they are fed with chow diet without or with trace amount of carotenoids. This leads to the hypothesis that BCO2 might play a crucial role to protect mitochondrial function other than just enzymatic function. The aim of this study is to explore the mechanism of how BCO2 maintains mitochondrial function by determining the impact of BCO2 on mitochondrial proteomics and respiratory activity.

2. Methods

2.1. Animals and animal Care

Male 129S6 WT and $BCO2^{-/-}$ mice at six weeks of age were selected and group-housed (3 mice/cage) in a controlled environment with a 12-h/12-h light/dark cycle. Mice were fed a regular chow diet (CD, 13.4% kcal from fat, #5001; LabDiet, St Louis, MO). All mice had free access to water and food throughout the study. Hypothalamic tissues were collected for laboratory analyses. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University.

2.2. Mitochondria isolation

Hypothalamus (60 mg) was homogenized (Qiagen tissuerupter) in 0.5 mL mitochondrial isolation buffer (210 mM mannitol, 70 mM sucrose, 5 Mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)), 1 mM ethylene glycol tetraacetic acid (EGTA) and 0.5% (w/v) fatty acid-free BSA, pH 7.2). The homogenate was centrifuged (600g, 5 minutes, 4 °C), and the supernatant was collected for further centrifugation (5000g, 5 min, 4 °C). The pellet was re-suspended in 1 mL mitochondrial isolation buffer and centrifuged again for 5 minutes (5000g, 4 °C). After removing the supernatant, the remaining mitochondrial pellet was re-suspended in 1 mL mitochondrial assay solution (1x MAS: 70 mM sucrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, 0.2% (w/v) fatty acid-free BSA). Total protein (ug/mL) was determined by the bicinchoninic acid assay kit (BCA assay, Pierce, Rockford, IL).

2.3. Mitochondrial proteome

Purified mitochondria (24 μg) were subjected to LC-MS/MS in Proteomics Core Facility of Oklahoma State University. Each genotype had 3 biological replicates, and 4 technical replicates. Spectral counting was applied for quantifying relative changes in protein abundance. The ratio of spectral counting between $BCO2^{-/-}$ mice and WT mice was performed to indicate the

fold change of protein expression level.

2.4 Ingenuity pathway analysis (IPA)

The metabolites identified in global metabolomic profiling with their p-value and fold change were uploaded to the IPA-Metabolomics platform. This platform is capable of integrating proteomics and transcriptomics data to identify significantly perturbed pathways, metabolic regulators, and novel biomarkers.

2.5. "Coupling assay" and "Electron flow" respiration analysis

Isolated hypothalamic mitochondria were used for "coupling assay" and "electron flow" respiration assay using the Seahorse XF96 Flux Analyzer (Seahorse Bioscience Inc., MA). All steps were conducted on ice unless otherwise stated. 4ug of mitochondria were loaded into each well of a XFe 96 cell culture microplate (Seahorse Bioscience Inc., MA). After centrifuging the microplate (2000 rpm, 10min, 4 °C), 70 uL pre-warmed (37 °C) 1x MAS, 90 uL pre- warmed (37 °C) coupling assay substrate (2x MAS with 10 mM pyruvate, 10 mM malate, and 10 mM succinate, pH=7.2) or electron flow substrate (2x MAS with 10 mM pyruvate, 10 mM malate, and 4 uM FCCP, pH=7.2) was gently added into each well. The plate was loaded into Extracellular Flux XFe 96 Analyzer (Seahorse Bioscience Inc., MA) to initiate the assay. ADP (4 mM, final), oligomycin (1.5 uM, final), FCCP (4 uM, final), and antimycin A (4 uM, final) were injected subsequently for coupling assay. For electron flow assay, rotenone (2 uM, final), succinate (10 mM, final), antimycin A (4 uM, final), and ascorbate /TMP [(10 mM) /(100 uM), final] were injected into each well. The oxygen consumption rate (OCR) during the whole process was measured.

2.6. Statistical analyses

Data were presented as mean ± SD. The results from respiration assay were processed by the XFe wave (Seahorse Bioscience Inc., MA), and displayed as oxygen consumption rates

(pMoles/min/well) vs. time. The differences in protein expression level and respiratory activity between WT and $BCO2^{-/-}$ mice were analyzed using student's *t*-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Altered hypothalamic mitochondrial proteomics in $BCO2^{-/-}$ mice

One hundred forty five mitochondrial proteins were identified by LC-MS/MS. To identify the difference in hypothalamic protein expression between WT and $BCO2^{-/-}$ mice, the cutoff point ratio ($BCO2^{-/-}$/WT) was set at ratio < 0.85 or ratio > 1.15, with p-value < 0.05 determined by two-tailed student's *t*-test. Among the 145 identified proteins, 30 hypothalamic mitochondrial proteins expressed differently in $BCO2^{-/-}$ mice compared to WT mice. Twenty-five of the proteins were down-regulated, while only five of them were up-regulated in $BCO2^{-/-}$ hypothalamus. The functional categorization of proteins differently expressed between genotypes was summarized in **Table 1**. Most of the proteins related to ETC, Krebs cycle, protein metabolism, and mitochondrial membrane were decreased in the hypothalamus of $BCO2^{-/-}$ mice.

Table 3 Functional categorization of proteins differently expressed in hypothalamic mitochondria of $BCO2^{-/-}$ mice vs. wild type (WT) mice

Gene symbol	Protein name	Ratio ($BCO2^{-/-}$ /WT)	p-values
ETC			
NDUFA6	NADH dehydrogenase 1 α subcomplex subunit 6	0.2692	0.0294
CYC1	Cytochrome c1, heme protein	0.5033	0.0169
NDUFS6	NADH dehydrogenase iron-sulfur protein 6	0.5414	0.0536
CISD1	CDGSH iron-sulfur domain 1	0.5856	0.003
ATP5C1	ATP synthase subunit γ	0.7023	0.0016
ATP5F1	ATP synthase F(0) complex subunit B1	0.6325	0.039
NDUFA9	NADH dehydrogenase 1α subcomplex subunit 9	0.655	0.0326
NDUFS7	NADH dehydrogenase iron-sulfur protein 7	0.7549	0.042
SDHA	Succinate dehydrogenase flavoprotein subunit	0.7639	0.046
NDUFA11	NADH dehydrogenase 1α subcomplex subunit 11	10.125	0.0303
TCA			
IDH3A	Isocitrate dehydrogenase NAD subunit α	0.6222	0.0264
PC	Pyruvate carboxylase	0.6532	0.0021
PDHA1	Pyruvate dehydrogenase E1 component subunit α, somatic form	0.7948	0.0023
MDH2	Malate dehydrogenase 2	0.8213	0.0451
β-oxidation			
HADHB	Mitochondrial trifunctional protein subunit β	0.8202	0.0125
LACS6	Long chain acyl-CoA synthetase 6 isoform 3	1.322	0.0406
Protein metabolism, folding, degradation			
HYOU1	Hypoxia up-regulated protein 1 protein	0.0465	0.0501
IVD	Isovaleryl-CoA dehydrogenase	0.2458	0.0377
MAOA	Amine oxidases (AO) flavin-containing	0.5055	0.0033
GOT2	Glutamic-Oxaloacetic Transaminase 2	0.828	0.0236
Membrane protein			
CKMT1	Creatine kinase 1	0.6126	0.0056
VDAC3	Voltage-dependent anion channel 3	0.6716	0.0027
VDAC1	Voltage dependent anion channel 1	0.7019	0.0158
SLC25A4	solute carrier family 25, member 4	0.707	0.0489
TOMM70A	Mitochondrial import receptor subunit TOM70	0.7562	0.0127
SLC25A5	solute carrier family 25, member 5	0.7739	0.0348
Misc			
CTNNB1	Catenin β-1	0.7007	0.0258
TPI1	Triosephosphate isomerase	1.6212	0.0179
PGK1	Phosphoglycerate kinase 1	3.65	0.015
CFL1	Cofilin-1	5.3636	0.0165

3.2. Canonical pathway analysis between WT and BCO2$^{-/-}$ mice

To further investigate the impact of BCO2 on hypothalamic mitochondrial metabolism and function, the canonical pathway analysis was performed **(Fig 7**). The top 12 altered pathways ranked by the p-value were mitochondrial dysfunction, oxidative phosphorylation, EIF2 signaling, fatty acid β-oxidation, valine degradation, phagosome maturation, TCA Cycle II (Eukaryotic), remodeling of epithelial adherens junctions, clathrin-mediated endocytosis signaling, GABA receptor signaling, isoleucine degradation, and Huntington's disease signaling. All 12 pathways were down-regulated in BCO2$^{-/-}$ mice hypothalamus compared to WT group.

3.3. Perturbed energy utilization caused by the deletion of BCO2

The impact of BCO2 on mitochondrial energy utilization pathway is shown in **Fig 8**. The Krebs cycle is the central metabolic pathway in aerobic organisms that generates a pool of chemical energy in the form of ATP, NADH, and FADH2. Five enzymes involved in Krebs cycle, including pyruvate carboxylase (PC), pyruvate dehydrogenase E1 component subunit α (PDHA1), succinate dehydrogenase flavoprotein subunit (SDHA), malate dehydrogenase (MDH2), and isocitrate dehydrogenase NAD subunit α (IDH3AI) were inhibited. The decreased PC and PDHA1 led to the inhibition of pyruvate converting to both oxaloacetate and acetyl-CoA, which may result in the overall down-regulation of Krebs cycle as indicated by canonical pathway analysis (**Fig 7**).

Fatty acid β-oxidation was also affected by BCO2. LCFA need to be activated by fatty acyl CoA synthetase to form long chain fatty acyl CoA before β-oxidation. The expression level of long chain acyl-CoA synthetase 6 isoform 3 (LACS6) was increased in BCO2-/- mice compared to WT mice. However, the expression of mitochondrial trifunctional protein subunit β (HADHB), which catalyzes the last three steps of mitochondrial β-oxidation of LCFAs was decreased in the hypothalamus of BCO2$^{-/-}$ mice.

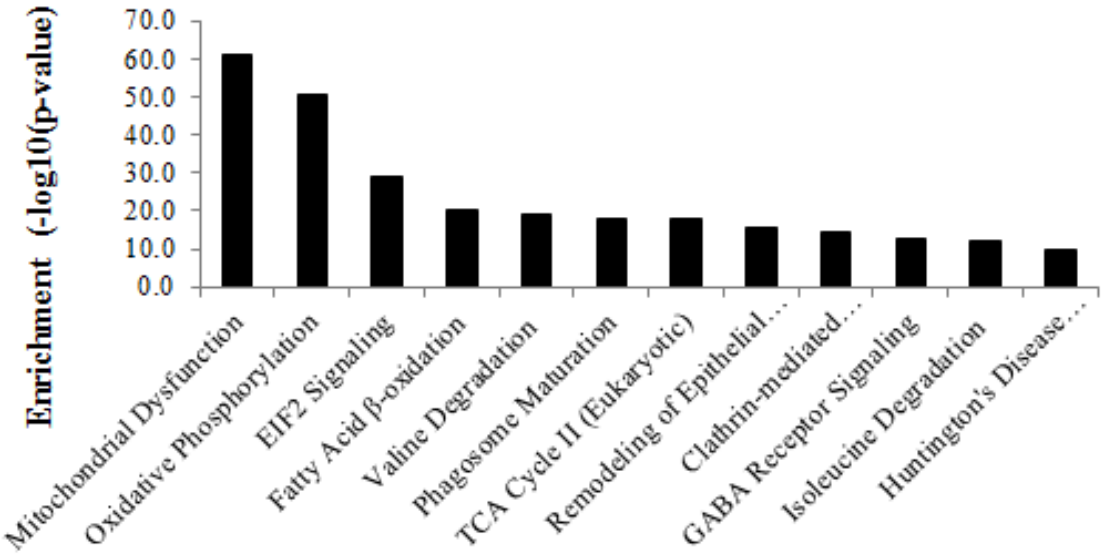


Fig. 7 The change in canonical pathways between WT and BCO2$^{-/-}$ mice. The list of proteins identified from LC-MS/MS was analyzed by ingenuity pathway analysis (IPA) platform. The top 12 altered pathways are shown in the figure ranked by the p-value.

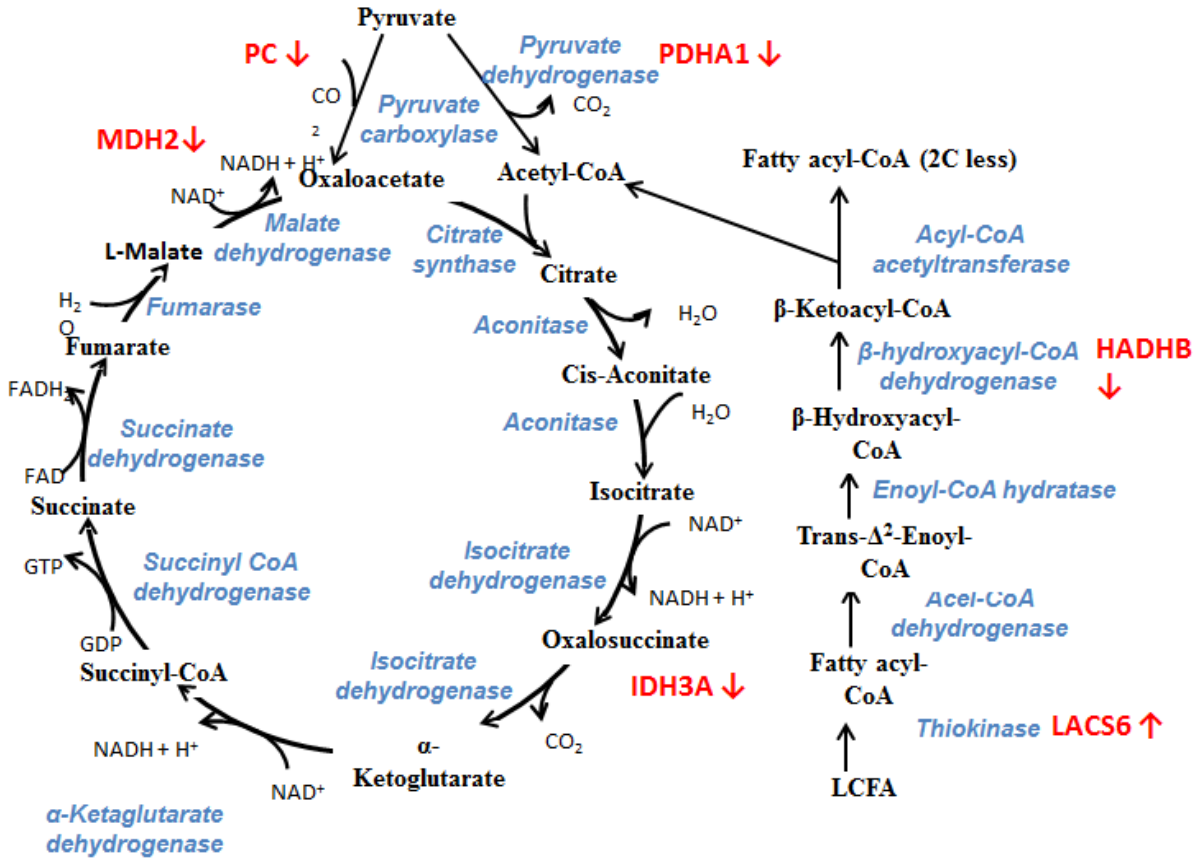


Fig. 8 Altered energy utilization caused by the deletion of BCO2. The proteins were identified from LC-MS/MS. Proteins expressed differently between genotypes related to Krebs cycle and fatty acid β-oxidation are shown in the figure. The cutoff point of ratio ($BCO2^{-/-}$ /WT) change was set at ratio < 0.85 or ratio > 1.15, with p-value < 0.05 determined by two-tailed student's *t*-test. PC: Pyruvate carboxylase; PDHA1:Pyruvate dehydrogenase E1 component subunit α; MDH2:Malate dehydrogenase; SDHA: Succinate dehydrogenase flavoprotein subunit; IDH3AI:socitrate dehydrogenase NAD subunit α; HADHB: Mitochondrial trifunctional protein subunit β; LACS6: Long chain acyl-CoA synthetase 6 isoform 3

3.4. Impact of BCO2 on ETC

The NADH and FADH2 generated during the Krebs cycle and LCFAs β-oxidation are oxidized through ETC/OXPHOS to produce large amount of ATP. Except for complex III, all the other complexes were affected by the deletion of BCO2 (**Fig. 9**). For complex I, the expression of NADH dehydrogenase 1 α subcomplex subunit 6 (NDUFA6) and subunit 9 (NDUFA9), as well as NADH dehydrogenase iron-sulfur protein 6 (NDUFS6) and 7 (NDUFS7) were decreased; while NADH dehydrogenase 1 α subcomplex subunit 11(NDUFA11) was increased. The SDHA in complex II was down-regulated. ATP synthase subunit γ (ATP5C1) and ATP synthase F(0) complex subunit B1 (ATP5F1) were down-regulated in $BCO2^{-/-}$ mice as well.

Besides the impacts on complexes of ETC, BCO2 knockout also led to the decreased expression of cytochrome C1 (CYC1), which plays an important role in transporting the electron from complex III to complex IV. In addition, the CDGSH Iron Sulfur Domain 1(CISD1) is the protein that plays a key role in regulating maximal capacity for electron transport and oxidative phosphorylation, it binds to the redox-active Fe-S cluster. Decreased CISD1 here indicated lower efficiency in KO mice.Furthermore, decreased expression of mitochondrial antioxidant MnSOD, which functions to detoxify superoxide into hydrogen peroxide and diatomic oxygen, was also observed in $BCO2^{-/-}$ mice ($p = 0.058$).

3.5. The impact of BCO2 on hypothalamic mitochondrial respiratory capacity

Functional analyses of isolated hypothalamic mitochondria were conducted to study the effect of BCO2 on hypothalamic mitochondrial respiratory capacity (**Fig 4**). The result from coupling assay showed that mitochondrial basal respiration rate, maximal respiration rate, and proton leak did not significantly change between WT and $BCO2^{-/-}$ mice. For the capacity of each ETC complex, the capacity of complex II was significantly decreased in $BCO2^{-/-}$ mice, while the capacity of complex I, IV, and V did not change between genotypes.

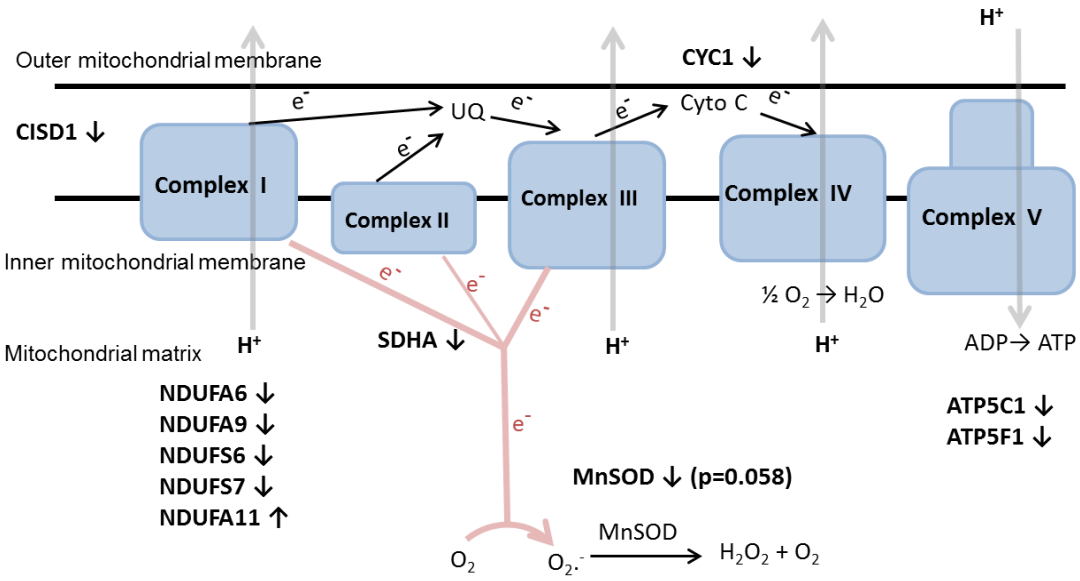


Fig. 9 The impact of BCO2 on electron transport chain. The proteins were identified from LC-MS/MS. The cutoff point of Ratio ($BCO2^{-/-}$ / WT) change was set at ratio < 0.85 or ratio > 1.15, with p-value < 0.05 (except MnSOD) determined by two-tailed student's t-test. UQ: ubiquinone; Cyto C: Cytochrome C;NDUFA6:NADH dehydrogenase 1 α subcomplex subunit 6; NDUFA9: NADH dehydrogenase 1 α subcomplex subunit 9; NDUFS6: NADH dehydrogenase iron-sulfur protein 6; NDUFS7: NADH dehydrogenase iron-sulfur protein 7; NDUFA11: NADH dehydrogenase1 α subcomplex subunit 11; SDHA: Succinate dehydrogenase flavoprotein subunit; CYC1:Cytochrome c1, heme protein; ATP5C1: ATP synthase subunit γ; ATP5F1:ATP synthase F(0) complex subunit B1; CISD1: CDGSH Iron Sulfur Domain 1; MnSOD: mitochondrial antioxidant manganese superoxide dismutase.

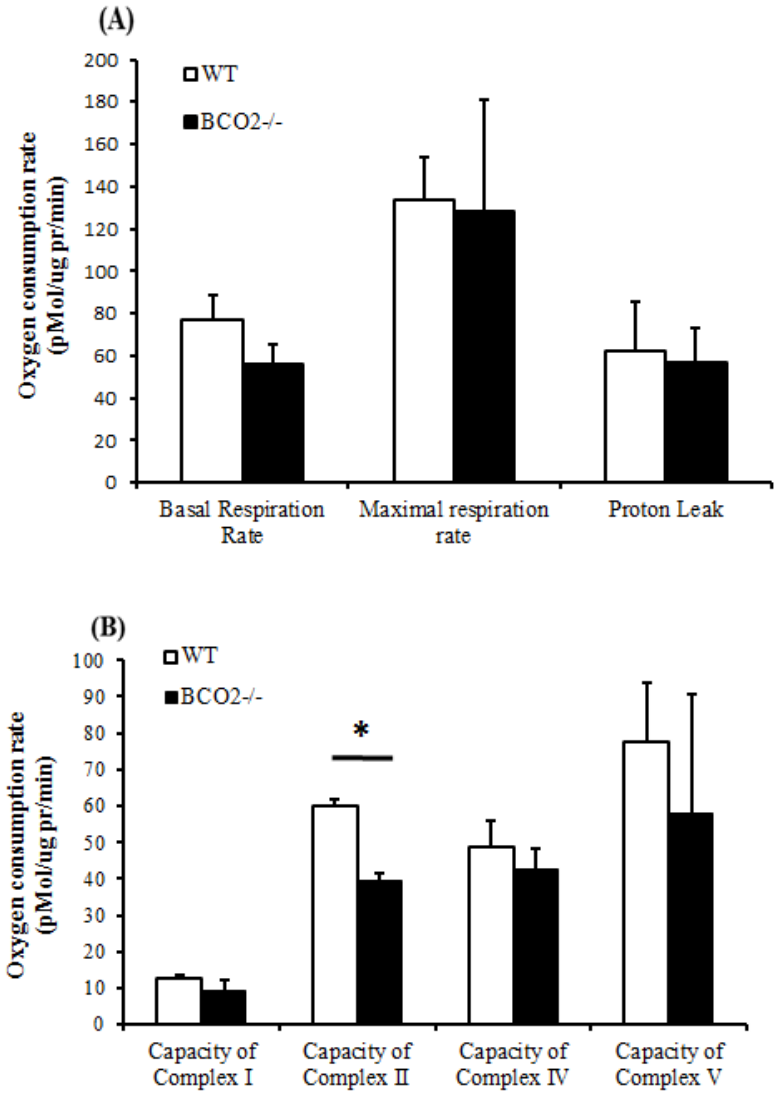


Fig. 10 Mitochondrial respiration rate (A) and capacity of each complex (B) between WT and BCO2$^{-/-}$ mice. Oxygen consumption rate was measured using isolated mitochondria from hypothalamus. n=12 technical replicates from 3 biological replicates.

4. Discussion

In this study, the impacts of BCO2 on mitochondrial proteomics and function in the hypothalamus were analyzed. The brain acts as the commander to regulate of energy homeostasis by adjusting both eating behavior and energy expenditure in response to many signals including nutritional status and circulating hormone levels [196]. Such regulation is a highly complex process, involving different brain regions ranging from cortex to brainstem, but the hypothalamus is the focus of peripheral signals and neural pathways that modulate energy homeostasis and body weight [196, 197]. As the major organelle producing ATP in the hypothalamus, hypothalamic mitochondria also affect appetite control and energy balance. Hypothalamic mitochondria dysfunction is found to be associated with anorexia in *anx/anx* mice [198]. It has been reported that BCO2 is important in maintaining mitochondrial function by reversing elevated oxidative stress and decreasing respiratory activity caused by the accumulation of carotenoids from the diet [23]. However, such protective effects were based on the enzymatic function of BCO2. Our earlier research found the decreased expression of BCO2 in both obese and diabetic mice even when the mice were fed a chow diet without carotenoids [20, 21]. Furthermore, BCO2 was found to be in the inner mitochondrial membrane, where complexes of the ETC/OXPHOS are located [31]. The evidence discussed above leads us to hypothesize that BCO2 may be important for mitochondrial function and overall energy homeostasis, rather than simply functioning as a carotenoid cleavage enzyme.

The first objective of the study was to illustrate altered mitochondrial proteomics caused by the absence of BCO2. Results showed that among the 30 significantly changed proteins, 25 of them were down regulated. The major altered pathways include ETC/OXPHOS, Krebs cycle, fatty acids β-oxidation, and protein metabolism.

The ETC/OXPHOS was the most affected in the absence of BCO2. One subunit of NADH

dehydrogenase, NDUFA11, was increased in BCO2$^{-/-}$ mice; however, the other four subunits (NDUFA6, NDUFA9, NDUFS6, NDUFS7) were all decreased. Besides the proteins involved in complex I, the succinate dehydrogenase (SDHA), and ATP synthase (ATP5C1 and ATP5F1) were also down-regulated in BCO2$^{-/-}$ mice compared to WT mice. Moreover, the CYC1, which is an electron carrier, was decreased by 49.7% in BCO2$^{-/-}$ mice. The superoxide anion radicals are by-products of ETC due to the leakage of electron to oxygen molecules in the mitochondrial matrix. The mitochondrial antioxidant manganese superoxide dismutase (MnSOD) is an important enzyme which detoxifies superoxide anions into hydrogen peroxide. Lower MnSOD levels may associate with higher susceptibility to oxidative stress and mitochondrial dysfunction resulting from elevation of ROS [199].

Krebs cycle generates most of the NADH and FADH2 that are used for ETC/OXPHOS. Stability of the cellular function of mitochondria requires the integrity of enzymatic reactions. Our data showed that BCO2$^{-/-}$ mice might have some problems with the regeneration of oxaloacetate, and the formation of acetyl-CoA, as revealed by the decreased PC and PDHA1 in BCO2$^{-/-}$ mice leading to less substrate entering the Krebs cycle. Less expressed in BCO2$^{-/-}$ mice were enzymes involved in the Krebs cycle, such as MDH2, which converts oxalosuccinate to α-ketoglutarate; SDHA that oxidizes succinate into fumarate; and IDH3AI that produces oxaloacetate and NADH from malate.

The canonical pathway analysis also revealed mitochondrial dysfunction in the hypothalamus of BCO2$^{-/-}$ mice. The function of the central nervous system is highly dependent on efficient mitochondria, due to the high energy demand of brain tissue [195]. Mitochondria are highly dynamic organelles that fuse and fission in response to environmental and energy demands. Besides the altered energy metabolism pathway, mitochondrial dysfunction also leads to increased oxidative stress and altered apoptosis. Reactive oxygen species (ROS) derived from mitochondrial

dysfunction are a major contributor to oxidative damage in the brain [200]. Mitochondria dysfunction is reported to play an important role in the ageing process and neurodegenerative disease, including Parkinson's disease [201, 202], Alzheimer's disease [203], and Huntington's disease [204, 205]. Furthermore, mitochondrial dysfunction and oxidative stress may be associated with abnormal brain function and psychiatric disorders, such as depression [206, 207].

The second aim of this study was to evaluate the impact of BCO2 on the respiratory activity of hypothalamic mitochondria. The absence of BCO2 led to significantly decreased capacity of complex II. In complex II, succinate dehydrogenase receives electrons to produce fumarate and FADH2, which then passes electrons to ubiquinone for the next steps of electron transport. The lower capacity of complex II is consistent with the decreased SDHA level in $BCO2^{-/-}$ mice as shown by the proteome data. The basal respiration rate, proton leak and capacity of complex I and IV did not change between WT and $BCO2^{-/-}$ mice. Since complex I, complex III, and complex IV are the proton pumps to form the proton gradient used for complex V to produce ATP, it is reasonable that the proton leak did not change between genotypes.

In summary, the BCO2 may have additional functions beyond that of carotene cleavage enzyme. It is important in maintaining mitochondrial function through the regulation of energy utilization, such as Krebs cycle, fatty acid β-oxidation, and ETC. Previously, most studies related to BCO2 were focused on the enzymatic function. This is the first research to assess the function of BCO2 on hypothalamic mitochondrial proteomics and respiratory activity in addition to its enzymatic function. Mitochondrial dysfunction has been considered as a major cause of aging and an underlying contributor to many neurodegenerative diseases [201, 203, 205, 208], as well as be associated with diabetes [52, 209], obesity [91], and even cancer [210]. Elucidating changes in mitochondrial proteins, pathways, and respiratory activities can provide better understanding of

the role of BCO2 in maintaining hypothalamic function. Building on this innovative approach, future research will examine the impact of BCO2 on hypothalamic metabolism and cell signaling.

CHAPTER V

CONCLUSION

Previously, most studies conducted on the function of BCO2 were focused on the enzymatic function. However, our lab found $BCO2^{-/-}$ mice exhibit higher food intake, and are more prone to obesity compare to wide type mice, even when mice were fed with chow diet, without carotenoids or just trace mount of carotenoids. The present study aims to demonstrated the mechanism how BCO2 impacts mitochondrial function and hypothalamic metabolome, and subsequent stimulates feeding behavior.

By using $BCO2^{-/-}$ mice model, we demonstrated inner mitochondrial membrane protein BCO2 does not only function as carotenoids cleavage enzyme. It is important to maintain mitochondrial function through the regulation of nutrients metabolism and utilization. The deletion of BCO2 lead to mitochondrial dysfunction, and significantly down-regulate of fatty acid β-oxidation, TCA Cycle and ETC pathways. BCO2 also protects mitochondria by maintaining MnSOD level to detoxify superoxide. For the mitochondrial respiratory activity, BCO2 is crucial to maintain the capacity of complex II of ETC.

Within the hypothalamus, the deletion of BCO2 leads to significantly lower level of glucose and LCFAs, such as oleic acid and DHA, which produce the nutrients depletion signal to stimulate appetite. The significantly lower circulating leptin level in $BCO2^{-/-}$ mice is another stimulus of appetite. Disrupted glucose and lipid metabolism, and inhibited bile acid metabolism leads to the consequent elevated oxidative stress and inflammation, which in turn contribute to the orexigenic phenotype. All these factors lead to a higher risk to develop into obesity of $BCO2^{-/-}$ mice.

In conclusion, the results presented here indicate that BCO2 contributes to hypothalamic mitochondrial function through its impact on the fatty acid oxidation, TCA cycle, and ETC/OXPHOS. The complete ablation of BCO2 could perturb energy metabolism, induce elevate oxidative stress, and impair cell signaling in hypothalamus. Current study is also the fundamental step of this new perspective. Our study also has some limitations. First, even though 477 metabolites have been measured by LC-MS/MS, some important metabolites, such as succinate, malonyl CoA, and LCFA-CoA cannot be detected due to technical limitation. This limitation prevented from being able to draw a more complete picture of how metabolism was altered by deletion of the BCO2 gene. Second, although low leptin levels provided us with a reasonable explanation for the increased appetite in $BCO2^{-/-}$ mice, we do not know why leptin levels decreased so dramatically in the $BCO2^{-/-}$ mice. Our next step in his research will be to focus on the impact of BCO2 on leptin production and leptin signaling to establish a better understanding of how BCO2 contributes to nutrient metabolism and overall body health.

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