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ChREBP- β regulates thermogenesis in brown adipose tissue

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Abstract

Brown adipose tissue (BAT) plays a critical role in energy expenditure by uncoupling protein 1 (UCP1)-mediated thermogenesis. Carbohydrate response element-binding protein (ChREBP) is one of the key transcription factors regulating *de novo* lipogenesis (DNL). As a constitutively active form, ChREBP- β is expressed at extremely low levels. Up to date, its functional relevance in BAT remains unclear. In this study, we show that ChREBP- β inhibits BAT thermogenesis. BAT ChREBP- β mRNA levels were elevated upon cold exposure, which prompted us to generate a mouse model overexpressing ChREBP- β specifically in BAT using the Cre/LoxP approach. ChREBP- β overexpression led to a whitening phenotype of BAT at room temperature, as evidenced by increased lipid droplet size and decreased mitochondrion content. Moreover, BAT thermogenesis was inhibited upon acute cold exposure, and its metabolic remodeling induced by long-term cold adaptation was significantly impaired by ChREBP- β overexpression. Mechanistically, ChREBP- β overexpression downregulated expression of genes involved in mitochondrial biogenesis, autophagy, and respiration. Furthermore, thermogenic gene expression (e.g. Dio2, UCP1) was markedly inhibited in BAT by the overexpressed ChREBP- β . Put together, our work points to ChREBP- β as a negative regulator of thermogenesis in brown adipocytes.

Key Words

- obesity
- lipogenesis
- metabolism
- gene targeting
- transcription factor

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Introduction

Obesity is a progressive metabolic disease that has become a global public health problem. As of 2015, approximately 2 billion people worldwide were classified as overweight and one-third of these were obese (Seidell & Halberstadt 2015, Gonzalez-Muniesa *et al.* 2017). Obese individuals have a high risk for developing complications, such as type-2 diabetes (T2D), cardiovascular disease,

and cancer. Metabolically, obesity results from energy imbalance developed by excessive energy intake relative to inadequate energy consumption, which is typically manifested by enlarged white adipose tissue (WAT), increased inflammatory response, and ectopic deposition of lipids (Hill *et al.* 2012). WAT is specialized for energy storage and also acts as a critical regulator for

metabolic homeostasis, which is primarily accomplished by the release of endocrine hormones (e.g. leptin, and adiponectin) (Friedman 2016). Whereas, brown adipose tissue (BAT) is specialized for energy expenditure by adaptive thermogenesis through the mitochondria and contributes to the maintenance of body temperature (Bartelt *et al.* 2011, Bartelt & Heeren 2014). Since the discovery of BAT in adult humans (Cypess *et al.* 2009, van Marken Lichtenbelt *et al.* 2009, Virtanen *et al.* 2009), the activation of BAT became an attractive therapeutic strategy for obesity. It has been postulated that cold-stimulated activation of BAT could counteract obesity and improve insulin resistance of patients with T2D (Yoneshiro *et al.* 2013, Hanssen *et al.* 2015).

BAT generates heat by an adaptive process called non-shivering thermogenesis (NST), which is mediated by uncoupling protein 1 (UCP1) (Porter *et al.* 2016). Upon acute cold stimulation, the sympathetic system is activated and releases catecholamine, which triggers fat mobilization and acutely activates UCP1 transcription, along with promoting mitochondrial biogenesis chronically through the induction of peroxisome proliferator-activated receptor gamma coactivator α (Pgc1 α) (Lowell & Spiegelman 2000, Chouchani *et al.* 2019). Long-term cold stimulation leads to metabolic remodeling in rodent BAT as well as WAT, which is characterized by increased mitochondrion number and UCP1 protein expression and decreased lipid droplet size, thereby rendering mice more adaptable to cold conditions. In addition, skeletal muscle is also a major alternate site of NST, and there is some interplay between these two pathways (Bombardier *et al.* 2013, Rowland *et al.* 2015, Bal *et al.* 2017).

Mitochondrial function is the basis for NST of brown adipocytes. Mature mitochondria are highly dynamic with fission and fusion processes, which are essential for mitochondrion renewal and activity. There are three GTPases involved in mitochondrial fusion process, that is, mitofusin 1 (MFN1), MFN2, and optic atrophic protein 1 (OPA1), while the fission process is mediated by mitochondrial dynamin-related protein (DRP) 1, which is activated by phosphorylation. Additionally, mitophagy also plays an important role in the maintenance of mitochondrial activity. Mitochondria may be damaged by reactive oxygen species, cell aging, and nutrient deficiencies, which result in depolarization and induce mitophagy. Damage-induced mitophagy can remove damaged mitochondria. This procedure is primarily driven by PTEN-activated PINK-I, which senses mitochondrial polarization and recruits E3 ubiquitin ligase (Parkin). Mitophagy in brown adipocytes can

be induced by cold exposure and regulated by thyroid hormone (Martinez-Lopez *et al.* 2016, Mottillo *et al.* 2016, Yau *et al.* 2019).

Free fatty acids are the main fuel to initiate thermogenesis and act as signaling molecules to promote the transcription of UCP1. Thus, lipolysis and fatty acid oxidation play important roles in BAT thermogenesis. Lipolysis in BAT provides fatty acids as initial fuel for thermogenesis, while lipolysis in WAT upregulates circulating fatty acids and provides additional fuel for long-term cold adaptation. Lipoprotein lipase (LPL) is dramatically upregulated during BAT activation to promote uptake of free fatty acids from circulation. β -oxidation can directly provide acetyl-CoA for thermogenesis. However, *de novo* lipogenesis (DNL) genes, such as *Acy*, *Fasn*, *ACC*, and *SCD1*, are upregulated in BAT by cold stimulation and correlate with UCP1 expression (Yu *et al.* 2002, Mottillo *et al.* 2014, Sanchez-Gurmaches *et al.* 2018). However, the functional significance of DNL in BAT remains unclear.

Carbohydrate response element-binding protein (ChREBP) is one of the key transcription factors regulating DNL in liver and intestine (Iizuka *et al.* 2004, Kim *et al.* 2016, 2017, Liu *et al.* 2017, Shi *et al.* 2020). Although ChREBP is also expressed in WAT and BAT, its biological functions in adipocytes are poorly understood. Due to distinct promoter-driven gene transcription, ChREBP has two subtypes, α and β , of which ChREBP- α is the dominant form in terms of expression levels and a capability of shuttling between the cytoplasm and the nucleus, while ChREBP- β is expressed at extremely low levels and acts as a constitutively active form (Herman *et al.* 2012). Upon activation, ChREBP- α can promote the expression of ChREBP- β , and ChREBP- β activates the transcription of downstream genes (Herman *et al.* 2012, Jing *et al.* 2016, Abdul-Wahed *et al.* 2017). However, the precise role of ChREBP- β in various tissues has not been defined due to its low expression levels and the difficulty of delineating the function of these two isoforms.

In this study, we took advantage of the Cre/LoxP approach to generate a mouse model with BAT-specific overexpression of the transgene ChREBP- β , hereafter designated BET- β . Unexpectedly, ChREBP- β overexpression caused a whitening phenotype of BAT with reduced mitochondrial contents and an impairment in thermogenesis upon cold exposure. Mechanistically, ChREBP- β inhibited the expression of genes involved in mitochondrial renewal and thermogenesis in BAT. Thus, our work suggests an important role of ChREBP- β in the thermogenesis of brown adipose tissue.

Materials and methods

Mouse experiments

All of the animal experiments were performed in accordance with the approvals obtained from the Naval Medical University Animal Ethics Committee (Shanghai, China). BAT-specific ChREBP- β overexpression mice were generated by crossing Rosa-ChREBP- β mice with UCP1-Cre mice. Mice were housed in a specific pathogen-free barrier facility under controlled temperature (23–25°C) with a 12 h light:12 h darkness cycle. The mice were fed *ad libitum* normal chow diet, otherwise, as indicated, a high-fat diet (HFD) with 60% fat in calorie.

Generation of ChREBP- β knockin mice

UCP1-Cre mice were generated by gene targeting as previously described, without compromise of UCP1 expression (Li *et al.* 2017). To generate ChREBP- β knock-in mice, we introduced a CAG promoter-driven mouse ChREBP- β expression cassette into the Rosa26 locus in ES cells with genetic background of C57BL/6J*129S3, in which a LoxP-flanked 3xStop sequence (LSL) was placed immediately upstream ChREBP- β cDNA as an expression blockage. The resultant germline was crossed onto the Flp transgenic mice to delete the Neo expression cassette, which was flanked by FRT sites and used for positive selection of the targeted ES cells. Then, Neo-deleted ChREBP- $\beta^{T/+}$ mice were crossed with UCP1-Cre mice to obtain ChREBP- β mice. The heterozygous ChREBP- β mice and control littermates were used in all the experiments.

Acute cold exposure

To mimic acute cold exposure, mice were placed in a cold incubator (4°C, without food) for up to 6 h. Body temperature was measured by a rectal probe. Body weight was measured before and after cold exposure. Mice were killed immediately after a 6-h cold exposure and tissues were isolated, weighed, and kept at –80°C after snap freezing in liquid nitrogen.

Long-term cold training

Mice were placed in a cold incubator with the temperature gradually decreased from 24°C to 4°C over 19 hours. Mice were then placed in 4°C chambers for up to 2 weeks. Body temperature was measured by a rectal probe. Body weight was measured before and after cold training. Mice were killed immediately after 2 h of fasting and tissues were isolated, weighed, and kept at –80°C after snap freezing in liquid nitrogen.

Glucose tolerance tests

Glucose tolerance tests were performed as previously described (Liu *et al.* 2017). Following 6 h of fasting, glucose tolerance was tested by oral gavages of glucose (2 g/kg of body weight; Sigma). Blood glucose was measured using a glucose glucometer by tail bleeding at 0, 15, 30, 60, and 120 min after oral gavage.

mRNA expression analysis

Total RNA was extracted from adipose tissue by a TRIZOL (Qiagen) method. The concentration and purity of total RNA were measured using spectrophotometry (NanoDrop; Thermo Scientific) with the ratios of A260 to A280 >1.8. cDNA was synthesized by a First Strand cDNA Synthesis Kit ReverTra Ace- α (Code No. FSK-100, Toyobo, Osaka, Japan). Quantitative PCR was performed on cDNAs by a fluorescent temperature cycler (Mastercycler Eppendorf, Eppendorf, Hamburg, Germany) with SYBR green and specific primers for target gene. All of the reactions were conducted in 96-well plates in a total volume of 10 μ L. All of the primers were synthesized by Jiali Bioscientific Company (Shanghai, China). In every plate, β -actin was used as the internal control.

Mitochondrial DNA quantification

Genomic and mitochondrial DNA was extracted from adipose tissue by the QIAamp DNA mini kit (Code No. 51304, Qiagen) method. Quantitative PCR was performed on total DNA by a fluorescent temperature cycler (Mastercycler Eppendorf, Eppendorf) with SYBR Green and specific primers for mitochondrial DNA (mtCOX2 and mtND1) and genomic DNA (β -globin). All of the reactions were conducted in 96-well plates in a total volume of 10 μ L. In every plate, β -globin was included as an internal control.

Western blots

Tissues were lysed in urea lysis buffer (25 mM Tris-HCl, 8 M Urea, 1% SDS, 1 mM EDTA, 0.7 M DTT, pH 7.4). After ultrasonic homogenization, lysates were centrifuged at 12,000 *g* for 15 min. The supernatant (i.e. total proteins) was separated by electrophoresis on 8–15% gradient SDS-PAGE gels, transferred onto PVDF membranes, and incubated with the primary antibodies listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article). Membranes were probed with horseradish peroxidase (HRP)-conjugated

anti-rabbit or anti-mouse secondary antibodies (1:5000, Vector Laboratories). Signals were generated using a Chemiluminescent Detection Kit (ECL Plus, Amersham Pharmacia Biotech) and visualized with a Luminescent Image Analyzer (LAS-4000mini, Fujifilm). Expression levels of each protein were normalized to α -tubulin or β -actin, which served as the loading controls.

Histological analyses

Pieces of the BAT fixed in 4% paraformaldehyde were embedded in paraffin and stained with hematoxylin and eosin. Immunohistochemical analysis of UCP1 was performed on BAT sections by overnight incubation at 4°C with rabbit polyclonal anti-UCP1 antibody (1:1000; ab10983) and subsequent incubation at 25°C with HRP-polymer conjugated anti-rabbit IgG (1:1000; ab6721) for 2 h before visualization with 0.05% DAB.

Electron microscope analyses

EM samples preparation and image collection were conducted according to the protocol described before and with some modifications (Su *et al.* 2019). In brief, mice were killed and perfused with 0.1 M sodium phosphate buffer (pH 7.4, 37°C) and then followed by pre-fixation solutions. BAT was cut and fixed in pre-fixation solutions for 2 h at room temperature. After rinsing with 0.1 M phosphate buffer, tissues were post-fixed with 2% osmium tetroxide. Samples were rinsed with high-purity water, followed by gradient dehydration with gradual acetone series and embedded in epoxy resin (60°C for 24 h), and then sectioned using Leica EM UC7 (about 60 nm thick) and placed on copper grids. Images were recorded on a FEI Tecnai G2 20 Twin electron microscope fitted with an EagleTM4k CCD digital camera (FEI).

Electromyography (EMG) experimental protocol

Surgical implantation of the electromyography electrodes was conducted according to the protocol described before (Feketa *et al.* 2013). In brief, mice were anesthetized with sevoflurane. A small area of the skin on the back was shaved, and spinotrapezius muscles were exposed. Electrode tips were inserted into spinotrapezius muscles and sutured to the muscles using 6-0 silk sutures. An additional reference electrode was placed under the skin 2 cm caudally from the recording electrodes along the midline. The skin was closed with 5-0 sutures. Then mice were placed in a 30°C incubator, and all experiments were performed 24 h

after surgery. Electromyography was recorded by a multi-channel physiological signal acquisition and processing system (RM6240BD, Chengdu Instrument Inc., Chengdu, China) at 30°C as baseline levels before measurement of cold-induced muscle shivering at 4°C for 30 min.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was performed as previously described (Liu *et al.* 2017). Briefly, BAT (100 mg) was crosslinked with 1% formaldehyde for 15 min at room temperature before stop by adding 0.5 M glycine. After micrococcal nuclease (M0247S, New England BioLabs) digestion and sonication, the sheared chromatin fraction was incubated with anti-ChREBP antibody (NB400-135, Novus Biologicals, 3 mg per reaction) in an ultrasonic water bath (30 min, 4°C) or normal rabbit IgG (#2729, Cell Signaling Technology) as negative control and followed by incubation with Dynabead-conjugated protein G (Invitrogen). Purified chromatin DNA was subjected to real-time PCR analysis with the primers for gene promoter, the sequence of which were listed in Supplementary Table 2. Mouse negative control primer Set1 (NC1, 71011, Active Motif, Carlsbad, CA, USA) was used as negative control. Four independent ChIP experiments were performed.

Statistical analysis

Different statistics methods were applied in this study. To compare the means between control and ChREBP- β mice, Student's *t*-test was applied. For the acute cold exposure tests, repeated measures ANOVA was used. For the multiple comparisons of means in different groups, the least significant difference (LSD) test were applied. Effects with $P \leq 0.05$ were considered to be statistically significant. The statistical analysis was conducted using the GraphPad Prism 6 and SPSS 17.0.

Results

Generation of BAT-specific ChREBP- β overexpressing mice

To investigate the potential role of ChREBP- β in BAT, we first examined its mRNA levels in normal C57BL/6 mice at different ambient temperatures. Quantitative RT-PCR analysis revealed that ChREBP- β mRNA levels were dramatically elevated in BAT at room temperature (24°C)

compared to the thermoneutral condition (30°C), which was accompanied by a slight increase in ChREBP- α mRNA levels (Fig. 1A). After the mice housed at room temperature were subjected to acute cold exposure at 4°C for 6 h, neither isoform of ChREBP was significantly changed at mRNA levels in BAT compared to those at room temperature. However, 2 weeks of chronic cold training resulted in an approximately three-fold increase in ChREBP- β mRNA levels in BAT compared to those at room temperature, while ChREBP- α mRNA levels were not significantly altered. These data suggest that ChREBP- β may play a role in BAT thermogenesis.

To evaluate the functional relevance of ChREBP- β in BAT, we generated BET- β mice by crossing Rosa-ChREBP β knock-in mice with UCP1-Cre mice (Li *et al.* 2017), which results in removal of the stop sequence by Cre/LoxP recombination, thereby allowing for conditional expression of the transgene ChREBP- β (Fig. 1B). Considering the nature of ChREBP- β low expression under physiological condition, we used the heterozygous BET- β mice with only one allele expressing the transgene in all the experiments. When housed at room temperature, heterozygous BET- β mice displayed a four to six-fold increase in ChREBP- β mRNA levels in BAT compared with control mice (Fig. 1C),

with no significant difference in inguinal WAT (iWAT) or epididymal WAT (eWAT) between the two genotypes. As a result, mRNA levels of the known ChREBP target genes involved in lipogenesis, such as Fasn, SCD1, and Elovl6, were significantly increased in BAT from BET- β mice compared with control mice (Fig. 1D), although their ChREBP- α mRNA levels were not significantly different. Remarkably, ChREBP- β protein was detected in the BAT from BET- β but not control mice by Western blot with a ChREBP-specific antibody, while ChREBP- α protein levels in BAT were similar between the two genotypes whether the mice were housed at room temperature or subjected to chronic cold training (Fig. 1E). Immunohistochemical analysis using anti-ChREBP antibodies showed that the nuclear staining of ChREBP was markedly increased and intensified in BAT from BET- β mice compared to control mice, which most likely resulted from the overexpressed ChREBP- β (Supplementary Fig. 1). Moreover, ChIP analysis of BAT revealed ChREBP binding to the promoters of target genes, for example, Fasn, and Thrpb, even at room temperature (Supplementary Fig. 2), which was consistent with their activated expression. These data suggest that we have successfully generated a mouse model with tissue-specific overexpression of ChREBP- β in BAT.

Figure 1

Generation of BET- β mice with ChREBP- β overexpression specifically in brown adipose tissue. (A) mRNA expression levels of ChREBP isoforms in BAT from adult male C57BL/6 mice on chow diet in response to different cold conditions. (B) Schematic demonstration for the generation of brown adipose tissue (BAT) specific ChREBP- β overexpression mice. A CAG promoter-driven ChREBP expression cassette with an internal loxP-flanked stop sequence as the transcriptional blockage was inserted at the Rosa26 locus by gene targeting in ES cells. The Neo expression cassette used for positive selection in ES cells was removed in germline by Flp/FRT recombination before backcrossing of ChREBP- $\beta^{+/+}$ mice with UCP1-Cre mice. In brown adipocytes, Cre-mediated deletion of the stop sequence upstream the ChREBP- β ORF allows for expression of the transgene ChREBP- β . (C) Specific overexpression of ChREBP- β mRNA in BAT from BET- β mice at room temperature ($n = 7$ per group). The data were shown after normalization with β -actin. iWAT, inguinal WAT; eWAT, epididymal WAT. (D) Upregulation of mRNA levels of the lipogenic target genes in BAT from BET- β mice ($n = 7$ per group). (E) Protein expression of ChREBP- α/β in BAT from BET- β and WT mice housed at room temperature or after 2 weeks of cold training ($n = 6$ per group). β -Actin was used as a loading control. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

BET- β mice failed to defend body temperature upon acute cold exposure

Given that DNL in adipose tissue benefits systemic insulin sensitivity, we first phenotypically analyzed glucose and lipid metabolism in BET- β mice. When housed at room temperature and fed a normal chow diet, neither male nor female BET- β mice exhibited significant differences in body weight or tissue mass of BAT, iWAT, eWAT, or liver compared with their control counterparts. In addition, blood glucose levels in fed or fasting state and glucose tolerance were not significantly affected by ChREBP- β overexpression in both genders (Supplementary Fig. 3A, B, C, D, E, F, G, H, I and J). Furthermore, BET- β and control mice were fed a high-fat diet (HFD) for 15 weeks and there was also no significant difference in body weight between the two groups (Supplementary Fig. 4A), suggesting that ChREBP- β overexpression in BAT did not affect energy homeostasis. However, HFD-fed BET- β mice showed an improvement in glucose tolerance in glucose tolerance test compared with their control mice (Supplementary Fig. 4B). These data suggest that overexpression of ChREBP- β in BAT may have some beneficial effect on glucose metabolism.

When housed at room temperature, BET- β mice had comparable rectal temperature as of control mice (Fig. 2A). To evaluate whether ChREBP- β overexpression affects

the thermogenic activity of BAT, we then exposed BET- β and control mice to acute cold condition (4°C) for up to 6 h without access to food. Interestingly, both male and female young BET- β mice failed to defend their body temperature under acute cold stress (Fig. 2B). During the first 2 h of cold exposure, BET- β mice maintained their body temperature. After the first 2 h, the body temperature of BET- β mice dropped rapidly, while the body temperature in control male mice remained normal throughout the cold exposure. Considering that both BAT thermogenic activity and UCP1 expression decrease with age (Yoneshiro *et al.* 2011), we tested cold tolerance on 1-year-old mice. Aged mice were less able to maintain body temperature after acute cold exposure than young mice, and BET- β mice were more susceptible to temperature loss (Fig. 2C). These data suggested a defect of BET- β mice in thermogenesis under acute cold condition.

The mechanism of body temperature maintenance in mice may be different in long-term cold training compared with acute cold exposure. Therefore, we also adapted mice to severe cold conditions (4°C) for 14 days. After long-term cold training, no difference of body weight or rectal temperature could be detected between BET- β and control mice (Fig. 2D and E). In addition, ChREBP- β overexpression also had no impact on the mass of adipose tissue or liver after long-term severe cold training (Fig. 2F), but iWAT weight ratio slightly increased in BET- β mice (Fig. 2G)

Figure 2

BET- β mice failed to defend body temperature upon acute cold exposure. Chow-fed BET- β and control mice at different age were housed at room temperature and subjected to cold exposure. (A) Rectal temperature at room temperature ($n = 9$ per group). (B and C) 10-week-old male or female mice (B) or 1-year-old male mice (C) were subjected to acute cold exposure at 4°C without food access for 6 h ($n = 5$ –6 per group). (D, E, F and G) Body weight (D), rectal temperature (E), tissues mass (F), and tissue to body weight ratios (G) of adult male mice after 2 weeks of chronic cold training at 4°C ($n = 6$ per group). Data are presented as mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$.

– the latter could be a compensatory effect. These data indicated that BET- β mice could maintain normal body temperature when adapted to room temperature or 4°C, but they failed to defend body temperature upon acute cold exposure without food access.

Characterization of BAT morphology and mitochondria of BET- β mice

To understand the mechanisms underlying the thermogenic defect with ChREBP- β overexpression in BAT, we histologically characterized the adipose tissue. In contrast to white adipocyte, brown adipocyte is characterized by smaller lipid droplets and abundant mitochondria. Interestingly, H&E staining revealed remarkable morphological changes in BAT from BET- β mice housed at room temperature or after long-term cold exposure, as manifested by enlarged lipid droplets compared with those from control mice (Fig. 3A). Electron microscope analysis revealed a reduction in the mitochondrion size in brown adipocytes from BET- β mice compared to the control; however, no significant changes

in mitochondrial architecture were observed (Fig. 3B). Furthermore, PCR analysis showed that the mitochondrial DNA (mtDNA) contents of mtND1 and Cox2 were decreased by 30% in BAT from BET- β mice housed at room temperature compared to control mice, suggesting a decrease in mtDNA copy number (Fig. 3C). Chronic cold exposure led to a robust increase in the mitochondrion contents of BAT from both BET- β and control mice and, to a much lesser extent, by acute cold exposure, but the differences between the two genotypes did not reach statistical significance (Fig. 3C). Taken together, the combination of enlarged lipid droplets and decreased mitochondrion contents strongly indicates a phenotype of BAT whitening in BET- β mice at room temperature.

To understand the mechanism underlying mitochondrial content alteration, we examined the effect of ChREBP- β overexpression on mitochondrial biogenesis. Western blot analysis revealed that BET- β mice housed at room temperature expressed similar levels of fission-related (i.e. Fis1 and DRP1) or fusion-related proteins (i.e. MFN2 and OPA1) in BAT as their control counterparts

Figure 3

Morphological and mitochondrial changes of BAT in BET- β mice. Chow-fed BET- β and control mice housed at room temperature were subjected to 6 h of acute cold exposure or 2 weeks of chronic cold training at 4°C. (A) H&E of BAT from BET- β and WT mice at room temperature and after 2 weeks of cold training. Scale bars, 100 μ m. The images were representative of three independent experiments. (B) Electron microscopic images showing the mitochondria of brown adipocytes from BET- β and WT mice at room temperature ($n = 3$ per group). Scale bars, 500 nm. The images were representative of three independent experiments. (C) Mitochondrial DNA contents of mtND1 and mtCOX2 in BAT from BET- β and WT mice ($n = 6$ per group). (D, E, F and G) Western blot analysis for the proteins involved in mitochondrial fission, fusion, and mitophagy (D and E) and respiratory chain reactions (F and G) in BAT from BET- β and WT mice at room temperature and after 6 h of acute cold exposure (D and F) or 2 weeks of cold training (E and G). Data are presented as mean $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

(Fig. 3D and E). Acute cold exposure did not significantly change Fis1 or MFN2 protein levels, but led to significant increase in the levels of phosphorylated DRP1 (p-DRP1) and the two isoforms of OPA1 in control BAT and had no significant effect on BAT from BET- β mice (Fig. 3D). Fis1 protein levels were decreased in BAT from BET- β mice after acute or chronic cold exposure compared with control group. In addition, p-DRP1 and OPA1 long isoform were also markedly declined in BAT from BET- β mice after chronic cold exposure compared with control group (Fig. 3E). These results indicate that overexpression of ChREBP- β might have inhibitory effects on mitochondrial fission and fusion upon cold exposure. Moreover, analysis of mitophagy-related proteins revealed that protein levels of PINK1, PTEN, and Parkin were similar in BAT between the two groups of mice housed at room temperature. Both acute and chronic cold exposure resulted in an elevation in the expression levels of the previously mentioned mitophagy-related proteins in control BAT compared to those at room temperature, but had no or attenuated effects in BET- β mice (Fig. 3D and E). These data suggest that ChREBP- β overexpression may impair mitophagy activity, thereby compromising mitochondrial renewal.

Mitochondrial respiration is dependent on the activity of electronic transport chain (ETC), which consists of five protein complexes (C-I to C-V). A proton gradient across the mitochondrial membrane created by C-I to C-IV drives heat production through UCP1-mediated uncoupling in brown adipocytes, rather than generating ATP through C-V (ATP synthase). We investigated if ETC composition was affected by ChREBP- β overexpression. To some different extents, a decrease in respiratory chain components, for example, CIII-UQCRC2, CIV-MTCO1, CII-SDHB, and CI-NDUFB8 occurred in the BAT of BET- β mice after acute and chronic cold exposure compared to control counterparts, of which C-IV subunits MTCO1 was most dramatically reduced, whereas CV-ATP5A was not significantly altered (Fig. 3F and G). These results suggest that ChREBP- β overexpression may inhibit mitochondrial respiration activity, leading to a defect in BAT thermogenesis.

Decreased expression of thermogenic genes in BAT from BET- β mice

To understand the mechanism underlying the impaired thermogenesis in BET- β mice, we examined the expression of thermogenic genes in BAT. When the mice were housed at room temperature, UCP1 expression was reduced by 38–50% in BAT from BET- β mice at the

mRNA and protein levels compared with control mice (Fig. 4A, B and C). Dio2, an important thermogenic gene which encodes deiodinase capable of catalyzing the conversion of thyroxine (T4) to the active thyroid hormone triiodothyronine (T3) (Bostrom *et al.* 2012), was downregulated in BAT by the overexpression of ChREBP- β by 70% and 30% at the mRNA and protein levels, respectively, (Fig. 4A, B and C). Acute cold exposure resulted in a robust increase in the mRNA levels of UCP1 and Dio2 in control BAT, but had marginal effect on UCP1 mRNA levels or attenuated effect on Dio2 mRNA levels in BET- β mice (Fig. 4A). However, UCP1 or Dio2 protein levels in BAT were not affected by acute cold exposure in either of the two genotypes compared to their counterparts at room temperature (Fig. 4B). On the other hand, chronic cold training did not significantly increase UCP1 or Dio2 mRNA levels in either of the genotypes (Fig. 4A), but Western blot revealed a marked elevation in their protein levels in control BAT compared to those at room temperature, suggesting that a transcription-independent mechanism may be involved. Of note, both UCP1 and Dio2 expression was markedly decreased at protein levels in BAT from BET- β mice after chronic cold training compared to that in control mice (Fig. 4C). Moreover, immunostaining also showed that UCP1 protein expression in BAT from BET- β mice was obviously weaker than that in control mice and hardly upregulated after chronic cold training (Fig. 4D).

To understand the transcriptional mechanisms about altered UCP1 expression in BET- β mice, we examined the expression Pgc1 α , which is a key transcriptional activator of UCP1 gene. At room temperature, Pgc1 α expression was relatively weak at the mRNA and protein levels and comparable between both genotypes (Fig. 4A and B). Acute cold exposure led to a robust increase in its mRNA and protein levels in control BAT and to a some less extent in BAT from BET- β mice. Chronic cold training did not significantly change the expression of Pgc1 α at the mRNA or protein levels in BAT from control or BET- β mice (Fig. 4A and C), suggesting that other mechanisms beyond Pgc1 α may be involved in the regulation of UCP1 expression. To exclude the possibility that ChREBP directly regulates UCP1 transcription, we performed ChIP analysis on BAT and found no significant binding of ChREBP protein to UCP1 promoter (Supplementary Fig. 2).

To determine whether ChREBP- β influences fuel supply to BAT thermogenesis, we examined expression of genes involved in glucose and lipid uptake and metabolism. Compared to control mice, BET- β mice exhibited a mild reduction in BAT mRNA levels

Figure 4

Downregulated expression of thermogenic genes in BAT in BET- β mice. Chow-fed BET- β and control mice were housed at room temperature before subjected to 6 h of acute cold exposure or 2 weeks of chronic cold training at 4°C. (A) mRNA levels of thermogenesis-related genes in BAT ($n = 6$ per group). (B and C) Protein levels of thermogenesis-related genes in BAT from BET- β and WT mice at room temperature and after 6 h of acute cold exposure (B) or 2 weeks of severe cold training (C). (D) Immunostaining of UCP1 in BAT from BET- β and WT mice at room temperature and after 2 weeks of cold training. UCP1 was developed with DAB. The images were representative of three independent experiments. Scale bars, 100 μ m. (E and F) Protein levels of lipases in BAT from BET- β and WT mice at room temperature and after 6 h acute cold exposure (E) and after 2 weeks severe cold training (F). Data are presented as mean $\pm$ S.E.M. * P < 0.05; ** P < 0.01.

of CD36, VLDLR, Fabp4, and CPT1 (carnitine palmitoyltransferase 1) under acute and/or chronic cold conditions, as well as a significant increase in Glut4 mRNA levels at room temperature and on acute cold exposure (Supplementary Fig. 5). In addition, LPL expression, which was markedly activated in control BAT at the mRNA and protein levels after cold exposure, was significantly downregulated in BET- β mice compared to control mice, while ATGL and HSL expression was comparable at the protein between the two genotypes (Fig. 4A, E and F).

Impaired WAT browning in BET- β mice in response to cold exposure

Considering that chronic cold exposure induces the browning of WAT in association with UCP1 activation, which contributes to adaptive thermogenesis, we next examined the potential effect of UCP1 activation-driven ChREBP- β overexpression on WAT browning induced by 2 weeks of cold exposure. ChREBP- β expression was mildly increased at the mRNA and protein levels in iWAT from BET- β mice compared to those in control mice, which was hard to detect in control group by Western blot

(Fig. 5A and B). Whereas, iWAT from the two genotypes showed no significant difference in the mRNA or protein levels of ChREBP- α or the targets genes Fasn and SCD1 (Fig. 5A, B and C). Of note, UCP1 and Dio2 expression was significantly downregulated at the mRNA levels in iWAT from BET- β mice compared to control mice (Fig. 5A), while their protein levels were decreased at higher magnitude in Western blotting (Fig. 5B). Immunohistochemical analysis also revealed weaker UCP1 staining in iWAT from BET- β mice compared to control mice (Fig. 5C). Furthermore, Western blot analysis showed that both groups expressed similar levels of proteins involved in mitochondrial fission (DRP1), fusion (MFN2, OPA1), or respiratory chain reaction in iWAT, while PINK1 levels were significantly decreased in BET- β mice compared to control mice, implying an impairment in mitophagy (Fig. 5D). On the other hand, there were no significant changes in the expression of ATGL, HSL, MGL, LPL, Ppar- α , or Ppar- γ at the mRNA and protein levels between the two genotypes (Fig. 5A and C). These results suggest that ChREBP- β overexpression in UCP1-positive cells may inhibit the browning of WAT in response to chronic cold exposure.

Figure 5

ChREBP- β overexpression impaired WAT browning in response to cold exposure. Chow-fed BET- β and control mice were subjected to chronic cold exposure at 4°C for 2 weeks before the analyses of their inguinal WAT. (A) mRNA levels of ChREBP and genes involved in thermogenesis and lipid metabolism. (B and C) Protein levels of ChREBP and genes involved in thermogenesis (B) and lipid metabolism (C). (D) UCP1 immunohistochemistry staining. Scale bars, 100 μ m. The images were representative of three independent experiments. (E and F) Expression levels of proteins involved in mitochondrial dynamics, mitophagy, and respiration. $n = 6$ per group. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$.

Unaffected muscle shivering and non-shivering thermogenesis in BET- β mice

Given the critical role of shivering in defending body temperature against acute cold exposure, we examined whether shivering might be affected by ChREBP- β overexpression in BAT. Electromyography (EMG) analysis

revealed comparable muscle shivering between BET- β and control mice during 30 min of acute cold exposure in terms of voltage range and peak number (Fig. 6A, B and C). On the other hand, skeletal muscle is also a major alternate site of non-shivering thermogenesis (NST) during cold adaptation (Bal *et al.* 2017), therefore we also evaluated the potential effect of ChREBP- β overexpression in BAT on

Figure 6

Characterization of shivering and non-shivering thermogenesis by skeletal muscle in BET- β mice. (A) The schematic illustration for the experimental protocol of electromyography (EMG) to record muscle shivering and representative electromyograms. (B and C) Voltage range (B) and peak number (C) of the electromyograms. (D and E) Expression levels of genes involved in non-shivering thermogenesis at the levels of mRNA (D) and protein (E) in soleus from BET- β and WT mice after a 12-h acute cold exposure ($n = 6$ per group). Data are presented as mean $\pm$ s.e.m. *** $P < 0.001$.

the expression of thermogenesis-related genes in muscle. qPCR and Western blot analysis of soleus revealed that BET- β and control mice had comparable expression of Serca1, Serca2, Plb, CamK2, and MFN2 at both mRNA and protein levels after 12 h of cold exposure and that the mRNA levels of Ryr1 and Sarcophilin (Sln) were not different between the two genotypes (Fig. 6D and E). Put together, these data suggest that ChREBP- β overexpression in BAT is unlikely to affect muscle thermogenesis by shivering or non-shivering mechanism.

Discussion

BAT thermogenesis is mediated by UCP1 uncoupling in mitochondria. The present study establishes an important role of ChREBP- β in BAT thermogenesis. We found that ChREBP- β rather than ChREBP- α was robustly activated upon both acute and chronic severe cold exposure, which is in consistence with a previous report (Sanchez-Gurmaches *et al.* 2018). Of importance, overexpression of ChREBP- β in brown adipocytes impaired BAT thermogenesis and conferred the mice more susceptible to body temperature loss under acute cold condition. Although skeletal muscle also plays an important role in the defense of body temperature loss, our preliminary EMG and gene expression analysis did not reveal any evidence about the potential influence of BAT ChREBP- β overexpression on skeletal muscle shivering or non-shivering thermogenesis at acute cold exposure. Further examination will be needed to ultimately address this issue in future investigation, which includes the phosphorylation and activation of CamKII and so on. Anyway, these findings suggest that the overexpressed ChREBP- β acts as a feedback regulator in cold-induced adaptive thermogenesis in BAT. Notably, ChREBP- β mRNA levels in the BAT from BET- β mice were increased by five-fold compared to control counterpart at room temperature or only by 1.7-fold when compared to chronically cold-exposed control mice. Therefore, this gain-of-function mouse model may reflect the physiological role of ChREBP- β in brown adipocytes upon cold exposure, which needs to be verified by loss-of-function approach in the future.

The study demonstrates that ChREBP- β regulates BAT thermogenesis at multiple levels. First, ChREBP- β overexpression caused the whitening phenotype of BAT, as evidenced by enlarged lipid droplets and reduced mtDNA copy number. BAT whitening is regarded as an indicator of BAT dysfunction in diet-induced obesity mice and UCP1 knockout mice (Shimizu *et al.* 2014,

Bond & Ntambi 2018, Kotzbeck *et al.* 2018). Second, many proteins involved in mitochondrial biogenesis, autophagy, and respiratory activity were downregulated in the BAT by ChREBP- β overexpression, implying a profound impact on mitochondrial function. A recent report demonstrates the important role of mitophagy in BAT thermogenesis (Yau *et al.* 2019). Lastly, ChREBP- β overexpression significantly inhibited the expression and cold-induced activation of relevant thermogenic genes (e.g. UCP1, Dio2, and Pgc1 α) as well as fuel uptake-related gene LPL. Mitochondrial UCP1 is essential for BAT thermogenesis (Chouchani *et al.* 2019). UCP1-null mice are cold intolerant due to impaired BAT thermogenesis, while heterozygous mice can retain normal body temperature when challenged with acute cold stress (Bond & Ntambi 2018). Besides, UCP1 deficiency is associated with a dramatic reduction of mitochondrial electronic transport chain components as well as mitochondrial dysfunction in BAT (Kazak *et al.* 2017). However, given the presence of half of UCP1 expression in the BAT, the defective thermogenesis of BET- β mice is not likely due to UCP1 downregulation alone, but rather, a UCP1-independent mechanism may be involved. It is interesting to note that UCP1 is dispensable for chronic cold adaptation (Keipert *et al.* 2017). Dio2 is another thermogenic protein catalyzing the conversion of T4 into active T3, and its deficiency impairs BAT thermogenesis despite normal UCP-1 expression and mtDNA copy number (de Jesus *et al.* 2001, Christoffolete *et al.* 2004). In BET- β mice, Dio2 expression was decreased at a higher magnitude than UCP1, suggesting that Dio2 downregulation might contribute to the defect of thermal homeostasis. Moreover, LPL, which catalyzes lipoprotein lipolysis to release fatty acids, is dramatically activated in BAT in response to cold stimulation (Klingenspor *et al.* 1996) and positively correlated with UCP1 expression and BAT thermogenesis in ANGPTL4 knockout (Singh *et al.* 2018). It is possible that the decreased LPL expression compromised fuel uptake of brown adipocytes from the circulation and resultant thermoregulation in BET- β mice. Taken together, it is most likely that the combination of the previously mentioned defects contribute to the impaired BAT thermogenesis in BET- β mice. The molecular mechanism by which ChREBP- β regulates the targets is under further investigation.

Our finding that ChREBP- β negatively regulates BAT thermogenesis is contrary to other reports. It has been reported that mild cold stimulation activates ChREBP-mediated DNL in BAT through the kinase AKT2 to optimize fuel storage and thermogenesis and that ChREBP- β expression positively correlates with UCP1

expression in human BAT (Sanchez-Gurmaches *et al.* 2018). However, the study did not address the direct effect of ChREBP on thermogenesis, without excluding the possibility that AKT2 promotes thermogenesis through ChREBP-independent mechanism. Katz *et al.* pointed out that T3 induces UCP1 expression through ChREBP, which can only be observed in the context of hyperglycemia or high glucose *in vitro* (Katz *et al.* 2018), suggesting that the other pathway is critically involved in regulating ChREBP and UCP1 expression. Both the studies focus on ChREBP pathway, without delineating the functions of ChREBP- β from ChREBP- α . Our study, for the first time, provides the direct evidence about the function of ChREBP- β in BAT thermogenesis, in which it serves as a negative regulator. One possibility for the discrepancy between our study and the other reports is that the two isoforms of ChREBP may have distinct functions. However, one caveat of our model is that ChREBP- β was constitutively overexpressed upon UCP1 activation, which may affect the early development of brown adipocytes. It was reported that expression of a truncated and constitutively active ChREBP- α in preadipocytes promotes PPAR γ activity and adipocyte differentiation (Witte *et al.* 2015). This concern can be addressed in the future by inducible overexpression of ChREBP- β at adulthood.

Our finding also demonstrates a role of BAT ChREBP- β in glucose homeostasis. ChREBP was originally identified as a transcription factor regulating DNL (Abdul-Wahed *et al.* 2017), and its expression levels in WAT are associated with metabolic health (Eissing *et al.* 2013, Kursawe *et al.* 2013). Both gain-of-function and loss-of-function studies show that ChREBP-regulated DNL in adipocytes has beneficial effects on metabolic homeostasis under the settings of overnutrition (Herman *et al.* 2012, Nuotio-Antar *et al.* 2015, Vijayakumar *et al.* 2017). Of note, ChREBP- β is regulated by mTOCR2 activity in WAT and positively correlates with DNL and insulin sensitivity (Tang *et al.* 2016). In BAT, DNL is reportedly coupled with lipolysis during chronic β 3-adrenergic receptor activation (Mottillo *et al.* 2014), but its role in thermogenesis has not been established. Our study demonstrated that overexpression of ChREBP- β in brown adipocytes increased DNL gene expression, along with improved metabolic performance under a high-fat diet, suggesting that BAT regulates systemic metabolism independently of thermogenesis. Interestingly, the non-thermogenic function of BAT has been reported in LKB knockout mice, which has imbalanced mitochondrial ETC components with the reduction of CIV-MTGO1 (Masand *et al.* 2018). Therefore, our finding supports the

notion that the regulation of glycose metabolism by BAT can be dissociated with its thermogenesis.

In summary, our study points to ChREBP- β as a negative regulator of BAT thermogenesis and provides an insight into the regulatory circuit of thermoregulation, which may help develop therapeutic approaches to obesity. Future investigations will be needed to fully understand the biochemical and molecular mechanisms about the regulation of thermogenesis by ChREBP.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0498>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Chunchun Wei designed and carried out the study, interpreted data, analyzed data, and drafted the article. Xianhua Ma performed the immunochemistry studies. Yuangang Zhu performed EM studies. Chenxin Wang and Xiaowei Chen analyzed the expressions of mitochondrial biology related genes. Kai Su, Shasha Qi, Junjian Lin, and Rui Yang performed the animal studies. Weiping Zhang and Weizhong Wang designed and conceived the experiments, reviewed the data, edited the article, and approved the version to be published.

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