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Intermittent cold exposure improves glucose homeostasis associated with brown and white adipose tissues in mice

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Abstract

Aims—The discovery of different shades of fat has been implicated in the pathogenesis of obesity-related metabolic disorders. However, the effects of early and intermittent exposure to cold temperature on systemic metabolic changes in adult life remain unclear.

Main methods—To elucidate the impact of cold temperature exposure on metabolic function of adipose tissues, we investigated the glucose homeostasis, activation of brown adipose tissue (BAT) and “browning” of white adipose tissue (WAT) in mice in response to intermittent cold exposure. Mice were exposed to 4 °C, 2 hours per day and 5 days per week, for 14 weeks. Glucose homeostasis was tested via intraperitoneal glucose tolerance test and insulin tolerance test; body fat mass was evaluated using *in vivo* magnetic resonance imaging; BAT activity was detected primarily by positron emission tomography/computed tomography; and WAT “browning” was evaluated using immunohistochemistry.

Key findings—Our results showed that 14-week cold exposure improved glucose tolerance and enhanced insulin sensitivity, reduced the relative weights of epididymal and retroperitoneal WAT, increased expressions of UCP1 and PGC1 α in subcutaneous adipose tissue.

Significance—Intermittent exposure to cold temperature in early life may improve systemic glucose homeostasis and induce WAT “browning”, suggesting that ambient cold temperature exposure may serve as a promising intervention to metabolic disorders.

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Introduction

The discovery of different shades of fat has been implicated in the pathogenesis of obesity-related metabolic disorders [1]. In addition to the storage of energy in the form of lipids, white adipose tissue (WAT) is well believed to contribute to the chronic inflammation during obesity by secreting so-called adipokines [2]. The metabolic disorders such as insulin resistance and type 2 diabetes mellitus are thought to arise from this chronic state of low-grade inflammation [3]. Unlike WAT, the primary function of brown adipose tissue (BAT) is to maintain core body temperature in response to cold stress by dissipating heat. This process, also known as thermogenesis, is primarily mediated by mitochondrial uncoupling protein (UCP) 1, which uncouples substrate oxidation from ATP production so that heat is generated [4]. The newly recognized existence of BAT in adult humans has prompted beneficial roles of this tissue in human health and disease development, particularly in obesity-mediated disorders [5-8].

In animal studies, chronic cold exposure has demonstrated to expand thermogenic capacity by increasing the number of brown adipocytes in BAT [9]; and by recruiting “beige” cells in WAT [10,11]. Although “beige” cells origin from WAT adipocyte precursor, there is robust overlap between “beige” and brown adipocytes in their expression of UCP1 and other thermogenic genes [12,13], indicating that stimulated “beige” cells could be involved in the regulation of energy balance like brown adipocytes. Peroxisome proliferator-activated receptor- γ (PPAR- γ) is the central regulator of adipose differentiation [14,15]. Activation of PPAR- γ by an agonist ligand is sufficient to trigger a full program of differentiation in fibroblastic cells, which includes morphological changes, lipid accumulation and expression of genes signature for fat cells. PPAR- γ -coactivator-1 α (PGC-1 α) is upregulated in brown adipocytes by cold exposure *in vivo*, or by β -adrenergic stimulation in isolated cells [16,17]; it is capable of inducing the key “browning” features in white adipocytes in culture or *in vivo* [16].

In addition to using lipids during non-shivering thermogenesis, BAT can also upregulate glucose uptake under cold stress. A recent human study showed that cold-activated BAT could increase glucose uptake by 12-fold [18]. BAT activation by β 3-agonist was shown to improve glucose tolerance and insulin sensitivity in genetically obese mice [19]. However, there are a few questions unanswered. For example, how early exposure to cold temperature impacts metabolic changes in adult life? If intermittent, short term exposure to cold temperature benefits overall body metabolism, especially WAT “browning”? To elucidate those impacts of cold exposure on metabolic function of adipose tissues, we hypothesized that intermittent cold exposure (ICE) from early age in mice improve glucose homeostasis associated with brown and white adipose tissues, and further investigated the glucose homeostasis, BAT activation and WAT “browning” in mice in response to ICE.

Materials and Methods

Animal care and ICE

Male C57 BL/6 mice (4-5 weeks old) were purchased from the Jackson Laboratories (Bar Harbor, ME). Animals were housed in two temperature-controlled chambers (Columbus

Instrument, OH) that comply with the guidelines for the care and use of laboratory animals. Mice were kept at 21-22 °C on a 12-hour light/dark cycle and fed with standard chow diet *ad libitum* at all times. After 3-week adaptation, mice were divided into two groups: control group and ICE group (n=10). For control group, mice were kept without temperature intervention; for ICE group, mice were exposed to cold exposure at 4 °C for 14 weeks (3-4 mice/cage; 2 hours per day; 5 days per week). The protocols and the use of animals were approved by and in accordance with the Ohio State University Animal Care and Use Committee.

Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT)

IPGTT and ITT assays were performed in consecutive days after 7-week and 14-week cold exposures. Mice were fasted overnight for IPGTT or 4.5 hours for ITT, followed by an intraperitoneal administration of dextrose (2 g/kg body weight) or insulin (0.5 U/kg body weight), respectively. Blood sample was collected from the vena caudalis and glucose levels were measured using an Contour Blood Glucose Meter (Bayer, Mishawaka, IN) at 0 (baseline), 30, 60, 90 and 120 minutes.

Magnetic resonance imaging (MRI)

Body fat mass (abdominal part) was detected after 14-week cold exposure following IPGTT and ITT tests using *in vivo* MRI, as described previously [21]. In brief, a Bruker 11.7T NMR system was operated at a proton frequency of 500 MHz with a gradient strength of 300 G/cm. Mouse was anesthetized with 2% isoflurane and placed in a 30-mm birdcage coil. A coronal spin-echo localizing sequence was used to identify both kidneys without respiratory or cardiac gating due to the fact that the abdomen is relatively free from motion artifact. Thirty contiguous, 1-mm thick axial slices, spanning from the superior pole of the uppermost kidney to the caudal aspect, were obtained using a spin-echo sequence with a 256×256 matrix size (pixel size, 117×117×1,000 μm³). Repetition and echo times for the T1-weighted images were about 1,000 and 13.0 ms, respectively, and the usage of 4 signal averages was provided for the best tissue contrast, with 17 minutes per scan of imaging time. Data analysis was performed using Image J software downloaded from the NIH website (Image J, <http://rsb.info.nih.gov/ij/>). Total abdominal volume, total adipose tissue, subcutaneous adipose tissue, and visceral adipose tissue of T1-weighted images were calculated as follows: the images were converted into two intensities with one corresponding to the adipose regions and the other to the remaining tissue. From these binary images, total adipose volume was equal to the volume of the intensity corresponding to the adipose region and total abdominal volume equal to the volume of both intensities combined. Next, the visceral adipose volume was calculated. The subcutaneous adipose tissue was calculated by subtracting the visceral adipose volume from the total adipose one. All volumes were normalized using the formula: normalized volume = $n \times \text{median number of images in a given population}$, where n is the number of slices measured in an individual T1-weighted image set.

Positron emission tomography/computed tomography (PET/CT)

BAT activities were evaluated after 14-week cold exposure following MRI evaluation via micro-PET/CT imaging, which was achieved with the Siemens Inveon Dedicated PET (dPET) System and Inveon Multimodality (MM) System (CT/SPECT) in the docked mode. A clinical package of ^{18}F -Fluorodeoxyglucose (FDG) (10 mCi) was purchased from Cardinal Health (Dublin, OH) and was diluted into the activity concentration of 250 $\mu\text{Ci}/100$ μl for each injection. Mice were fasted for 4 hours before one dose of ^{18}F -FDG was administered and were remained for one additional hour post FDG injection. Mice were then scanned with a small animal-dedicated micro-PET/CT system. During the scanning, mouse was placed into an anesthesia induction chamber with 2% isoflurane in oxygen. Post-imaging analysis was conducted using Inveon Research Workplace software and FDG uptake in BAT was quantified.

Tissue collection and histology

Mice were weighed before and after 14-week cold exposure to calculate body weight gain. After cold exposure, mice were sacrificed and adipose tissues from different depots were excised, rinsed with icy saline and weighed. Interscapular BAT (iBAT) and subcutaneous WAT (sWAT) were fixed in 4% formaldehyde overnight. Tissues were then dehydrated in a series of graded ethanol, cleared in xylene and embedded in paraffin. Samples were sectioned to 5 μm slides by a microtome and stained with hematoxylin and eosin (H&E). The images were photographed under a microscope (Zeiss 510 META, Jena, Germany), and were analyzed for calculation of brown adipocyte size and white adipocyte diameter using Image J software.

Immunohistochemistry (IHC)

Tissues of sWAT were fixed in 10% neutral buffer formalin and IHC staining was conducted as previously described [22]. In brief, the embedded adipose tissue was cut into 8- μm thick sections, fixed with ice-cold methanol, and blocked with 1.5% horse serum. The slides were then overnight incubated with primary antibodies at 4 °C. After 2-hour incubation with appropriate peroxidase-conjugated secondary antibodies, the stain was developed using fast 3, 3'-diaminobenzidine tablet sets (D4293; Sigma, St. Louis, MO). The sections were then counterstained with hematoxylin and examined by light microscopy. The primary antibodies were rat anti-mouse UCP1 and rat anti-mouse PGC1 α (Abcam, Cambridge, MA). Quantification of positive staining was performed from 3 to 4 slides per mouse in each group, and all measurements were conducted in a double-blinded manner by two independent investigators.

Western blot analysis

iBAT and sWAT were homogenized using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific). The same amounts of protein (20 μg) were separated with SDS-PAGE in 10% polyacrylamide gel, and then transferred to PVDF membranes (Biomad, Hercules, CA). After blocked in 5% bovine serum albumin in PBS-Tween 20 for 1 hour, the membranes were incubated with rat anti-mouse UCP1 or PGC1 α (Abcam, Cambridge, MA) overnight at 4°C, followed by the incubation of appropriate horseradish peroxidase-linked

secondary antibodies for 2 hours at room temperature. The membranes were detected with chemiluminescence (Thermo Scientific, Rockford, IL). Protein bands on the film were scanned and the densities were calculated using Image J software.

Statistical analysis

Data were presented as mean $\pm$ S.E. unless otherwise stated. Unpaired *t*-tests were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). In all cases, a *p* value less than 0.05 was considered to be statistically significant.

Results

Effect of ICE on glucose homeostasis in mice

To test whether cold exposure improves glucose homeostasis systemically, we performed IPGTT and ITT in those mice at 7-week and 14-week cold exposure, respectively, and the examinations at midterm week 7 served as a “time course” evaluation to see how mice responded to the exposure in circulatory glucose level. Our data showed that 7-week cold exposure failed to induce significant difference in glucose tolerance or insulin sensitivity between two groups (Fig. 1, A and B). However, after 14-week exposure, mice exhibited improved glucose tolerance and enhanced insulin sensitivity compared with control group (Fig. 1, C and D).

Effect of ICE on fat mass and distribution

Abdominal fat mass and distribution were evaluated by MRI. Cold exposure failed to induce significant changes in total abdominal fat content compared with control mice (Fig. 2, A and B). With respect to abdominal fat distribution, we found no difference in both visceral and subcutaneous fat contents between ICE and control groups (Fig. 2, C and D). In addition, body weight gain and relative fat weights were calculated to evaluate the effects of ICE. Our data revealed that cold exposure did not affect the body weight gain, relative weights of iBAT and sWAT (Fig. 3, A-C); but significantly reduced the relative weights of epididymal white adipose tissue (eWAT) and retroperitoneal white adipose tissue (rWAT; Fig. 3, D and E).

Effect of ICE on iBAT

To detect the effects of ICE on iBAT, we first calculated brown adipocyte numbers via H & E staining and observed more brown adipocytes in exposed mice compared with control mice (Fig. 4, A and B). Then BAT activity was examined by quantifying the uptake of ^{18}F -FDG using PET/CT; the intensity of ^{18}F -FDG was found to be upregulated about 8-fold in cold-treated mice (Fig. 4, C-E). UCP1 functions as a key mediator in thermogenesis via uncoupling of oxidative phosphorylation. In our study, UCP1 levels were detected via western blot. Although there was no significant difference in UCP1 levels between ICE and control groups, a substantial trend towards an increase was observed in exposed mice (Fig. 4, F and G).

Effect of ICE on sWAT

To evaluate the effects of ICE on sWAT, average cell size was quantified via H & E staining. The results showed that cold exposure caused a decrease in average cell size compared with control group (Fig. 5, A and B). IHCs were performed to evaluate the “browning” of sWAT. Our data indicated that cold exposure recruited more UCP1 positive cells (Fig. 5, C and D) and PGC1 α positive cells (Fig. 5, E and F) within sWAT. These findings were verified by Western blot experiments, showing that cold exposure upregulated the expressions of UCP1 and PGC1 α compared with control mice (Fig. 5, G-I).

Discussion

In the present study, we investigated the effects of ICE on glucose homeostasis in a mouse model that is commonly used in metabolic syndrome research. Our data demonstrated that 14-week ICE from early age improved systemic glucose homeostasis, increased BAT activation, and induced WAT “browning” in tested mice. This is significant and novel in terms of exposure to cold temperature from very early age (right after weaning in mice) impacts metabolic changes in adult lives. Furthermore, intermittent, short term exposure to cold temperature benefits overall body metabolism, especially WAT “browning” since no need or it is impractical to expose to cold in a sustainable manner due to our well advanced, air-conditioned (or even climate-controlled) living environment.

It was believed that BAT regressed with aging and was completely disappeared in adulthood in human [23]; and the studies of BAT had been limited mostly to rodents. Recent findings that BAT exists in human adults have opened a new chapter about the roles of BAT in human disease development [6-8]. BAT has currently been implicated as a promising target to treat obesity-mediated diseases. Both human and animal studies have suggested that BAT activation could improve glucose homeostasis [18,19]. BAT is heavily innervated by sympathetic nerves, and is responsible for non-shivering thermogenesis during cold stress. UCP1 is the major molecule involved in cold-mediated thermogenesis through uncoupling substrate oxidation from ATP production. Indeed, compared with wild-type mice, UCP1^{-/-} mice have reduced body temperature during cold exposure [24]. In our study, we showed that ICE upregulated BAT activity and UCP1 levels, indicating that cold-induced BAT activation may play a significant role in improving glucose homeostasis in stressed mice.

Recently, it was discovered that certain stimuli, such as chronic cold exposure, hormonal stimuli and β -adrenergic stimulation, can induce “browning” process in certain depots of WAT. These inducible brown adipocytes, also known as “beige” cells, possess many similar features of BAT, e.g. the presence of multilocular lipid droplets, multiple mitochondria and UCP1 expression. UCP1 and other genes related to mitochondrial biogenesis mediate adaptive thermogenesis and energy expenditure in response to cold stress [25]. PGC-1 α , a coactivator for PPAR- γ , is upregulated in brown adipocytes by cold exposure and is capable of inducing the key browning features in white adipocytes in culture or *in vivo* [16]. In a study with rats that were exposed to 4 C° for 4 days and acutely treated with insulin with/without an antisense oligonucleotide to PGC-1 α , cold exposure promoted a significant increase of PGC1 α and uncoupling protein-3 expression, increased glucose uptake and GLUT4 expression and membrane localization in type I and type II fibers of gastrocnemius

muscle, promoted significantly lower insulin-induced tyrosine phosphorylation of the insulin receptor (IR) and Ser473 phosphorylation of acute transforming retrovirus thymoma (Akt) and an insulin-independent increase of Thr172 phosphorylation of adenosine 5'-monophosphate-activated protein kinase (AMPK), and inhibition of PGC-1 α expression in cold-exposed rats by antisense oligonucleotide treatment diminished glucose clearance rates and reduced GLUT4 expression and membrane localization, suggesting a pathway of GLUT4 expression and subcellular localization independent of the IR and Akt activities but dependent on AMPK in cold-induced hyperexpression of PGC-1 α in skeletal muscle glucose uptake [26]. Later on, PGC-1 α ^{-/-} mice failed to maintain core body temperature following cold exposure, developed hepatic steatosis due to a combination of reduced mitochondrial respiratory capacity and an increased expression of lipogenic genes following short-term starvation, demonstrating PGC-1 α necessary for appropriate adaptation to the metabolic and physiologic stressors [27]. Our data further confirmed the prevailing evidence showing that ICE induces “browning” of WAT through upregulation of PGC-1 α , which may contribute to the effects of cold stress on glucose homeostasis, and further application in the prevention and treatment of metabolic syndromes [28].

The protocol design of the cold exposure was from the consideration that a time period that is reasonable but also represents real people's lives, which is a scenario of people who may live in a cold region for a decade in their life time, equivalent roughly 3-4 months in mice (2 to 2.5 years life expectancy) [29]. A 2-hour exposure time period was chose that had sufficient exposure time while at the same time avoiding “excessive” exposure to cold. As well-recognized, humans in our current societies are rarely directly exposed to outdoor extreme temperatures, and our civilization even has this as one of the purposes (clothes, houses, and air condition) [30]. Therefore, it is very difficult, if not completely impossible, to have large clinical or epidemiological studies that demonstrate sustainable effects of cold exposure in the developed regions. Even though, one of the early studies conducted in northern Finland indicated that working in the cold could retain BAT in human adults [31]. Another clinical study at Harvard from 3640 consecutive PET-CT scans in 1972 patients demonstrated that the probability of BAT detection was inversely correlated with outdoor temperature, body-mass index, and circulatory glucose level [8]. A recent clinical study with twelve males at University of Texas found that cold exposure significantly increased resting energy expenditure, whole-body glucose disposal, plasma glucose oxidation, and insulin sensitivity in the BAT positive group (seven males) only when compared to BAT negative group (five males), supporting the notion that BAT may function as an antidiabetic tissue in humans [32]. The finding that cold exposure improved glucose homeostasis is consistent with previous studies [33,34]. However, uncertainty exists regarding the exact role of cold stress in improving insulin resistance. In contrast to our findings, some studies suggest that cold stress exaggerated local insulin resistance and diet-induced obesity [27,35]. The conflicting results may be caused by different background strain, different baseline of body weight, or different protocol of cold exposure. These key elements must be considered to further evaluate the effects of cold stress on glucose homeostasis.

In conclusion, our data demonstrate that ICE from early age improves systemic glucose homeostasis, induces activation of iBAT and “browning” of sWAT. These findings have

significant implications in regulating energy expenditure and coping with global pandemic of obesity and metabolic disorders.

Conclusion

In summary, this study demonstrates that intermittent cold exposure from early age improved systemic glucose homeostasis, increased BAT activation, and induced WAT “browning” in a murine model.

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Fig. 1. Effects of cold exposure on glucose homeostasis in mice

(A) IPGTT in overnight fasted mice after 7-week cold exposure; (B) ITT in 4.5 hours fasted mice after 7-week cold exposure; (C) IPGTT in overnight fasted mice after 14-week cold exposure; and (D) ITT in 4.5 hours fasted mice after 14-week cold exposure. Bar graphs indicate the area under curve of the respective IPGTT or ITT curves. RT: room temperature; IPGTT: intra-peritoneal glucose tolerance test; ITT: insulin tolerance test; and AUC: area under curve. * $p < 0.05$ compared to control group. $n = 10$.

Fig. 2. Effect of 14-week cold exposure on fat mass and distribution in mice
(A) representative MRI images of abdominal mass; (B) percentage of total fat content in total abdominal mass; (C) percentage of visceral fat in total abdominal mass; and (D) percentage of subcutaneous fat in total abdominal mass. RT: room temperature. n = 6.

Fig. 3. Body weight gain and relative fat weights

(A) body weight gain; (B-E) ratio of noted fat to body weight. iBAT: interscapular brown adipose tissue; sWAT: subcutaneous fat (inguinal fat); eWAT: epididymal fat; and rWAT: retroperitoneal fat; RT: room temperature. * $p < 0.05$ compared to control group. $n = 8-10$.

Fig. 4. Effects of 14-week cold exposure on iBAT

(A-B) representative images of iBAT by H&E staining and quantitated brown adipocyte number, $n = 8$; (C) animal position during PET/CT, cross indicates the area corresponding to iBAT; (D-E) representative PET/CT images and quantification of the intensity of FDG, $n = 6$; (F-G) protein bands and quantitated column bars, $n = 3$. RT: room temperature; UCP1: uncoupling protein 1; and GADPH: glyceraldehyde-3-phosphate dehydrogenase. * $p < 0.05$ compared to control group.

Fig. 5. Effect of 14-week cold exposure on sWAT

(**A-B**) representative images of sWAT by H& E staining and quantitated cell size, $n = 5$; (**C-D**) representative IHC images stained with UCP1 antibody and quantitated UCP1 positive cells, $n = 5$; (**E-F**) representative IHC images stained with PGC1 α antibody and quantitated PGC1 α positive cells, $n = 4$; (**G-I**) protein bands and quantitated column bars, $n = 3$. RT: room temperature; UCP1: uncoupling protein 1; PGC1 α : Peroxisome proliferator-activated receptor- γ -coactivator-1 α ; and GADPH: glyceraldehyde-3-phosphate dehydrogenase. * $p < 0.05$ compared to control group.