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Exercise-induced beige adipogenesis of iWAT in Cidea reporter mice

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Obesity is caused by an imbalance between energy intake and energy expenditure. Exercise is attracting attention as one of the ways to treat obesity. Exercise induces 'beige adipogenesis' in white adipose tissue, increasing total energy expenditure via energy dissipation in the form of heat. Also, beige adipogenesis can be induced by treatment with a beta-adrenergic receptor agonist. We developed a Cidea-dual reporter mouse (Cidea-P2A-Luc2-T2A-tdTomato, Luciferase/tdTomato) model to trace and measure beige adipogenesis *in vivo*. As a result, both exercise and injection of beta-adrenergic receptor agonist induced beige adipogenesis and was detected through fluorescence and luminescence. We confirmed that exercise and beta-adrenergic receptor agonist induce beige adipogenesis in Cidea-dual reporter mouse, which will be widely used for detecting beige adipogenesis *in vivo*. [BMB Reports 2022; 55(4): 187-191]

INTRODUCTION

White adipocytes turn into beige adipocytes following treatment with a beta-adrenergic receptor agonist or cold exposure (1, 2). The phenomenon is known as 'browning', which contributes to increased total energy expenditure, whereby energy is dissipated in the form of heat (3, 4). Browning triggers cell death-inducing DNA fragmentation factor-like effector A (Cidea) expression along with other thermogenesis genes (5). Cidea is a member of the CIDE family of proteins. It is a lipid-droplet-associated protein that is highly expressed in brown adipose tissue promoting the enlargement of lipid droplets, which are dynamic, ubiquitous organelles specialized for storing neutral lipids (5, 6).

Exercise is an important strategy to treat cardiovascular diseases and type 2 diabetes mellitus among several diseases and health conditions (7, 8). It is well known that exercise induces dynamic changes in the whole body and positively modulates the health status (9, 10). Exercise induces browning of white adipose tissue via various secreted factors and mechanisms (11).

We investigated the role of exercise in inducing browning using Cidea reporter mice because Cidea is one of the important marker genes associated with browning. Transgenic (TG) Cidea mice contain a dual reporter system (Cidea-P2A-Luc2-T2A-tdTomato, Luciferase 2/tandem-dimer Tomato) (Supplementary Material). A multicistronic transcript was generated under the promoter of Cidea, and the translated protein was split into CIDEA, Luc2, and tdTomato proteins via 2A self-cleaving peptides (P2A, T2A). Bioluminescence and tdTomato fluorescence is upregulated by increasing the Cidea gene expression during browning of white adipocytes. Therefore, the parts affected by browning can be easily identified based on the luminescence of luciferase and the red color of tdTomato.

RESULTS

Activation of brown adipose tissue by exercise

We analyzed the expression of brown adipocyte marker genes in BAT. Exercise significantly increased the levels of UCP1, Cidea, and PGC-1α mRNA (Fig. 1A). The increased expression of Cidea following exercise was also demonstrated by bioluminescence. We acquired *in vivo* images to determine exercise-induced activation of brown adipose tissue. Sedentary TG mice expressed bioluminescence around the interscapular region (Fig. 1B). However, TG mice exercising for 8 weeks on a running wheel exhibited enhanced bioluminescence-expressing area in BAT (Fig. 1C). It was clear that TG mice expressed stronger bioluminescence in BAT after exercise than sedentary TG mice (Fig. 1D). However, wild-type mice did not show bioluminescence whether or not they performed wheel running exercises (Fig. 1E). BAT derived from control and exercise TG mice was cryosectioned and imaged to determine exercise-induced activation of tdTomato expression (red). tdTomato was

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expressed in BAT derived from both sedentary TG (Fig. 1F) and exercised TG mice (Fig. 1G). No expression of tdTomato was detected in WT mice in the sedentary group (Fig. 1H) and even after exercise (Fig. 1I).

Activation of brown adipose tissue was induced by CL-316,243 injection

We obtained *in vivo* images to determine the activation of BAT following CL injection. Both TG mice injected with saline and CL expressed bioluminescence in BAT. However, TG mice injected with CL showed stronger bioluminescence in BAT (Fig. 2A, B). The difference was clear when the two mice were compared with each other (Fig. 2C). BAT was activated by CL treatment. However, wild-type mice showed no bioluminescence in both CON and CL groups (Fig. 2D). BAT derived from TG and WT mice was cryosectioned and imaged to determine tdTomato expression (red) and CL-induced activation. tdTomato was highly expressed in BAT derived from both control and CL-treated mice and detected in the whole area of BAT (Fig. 2E, F). The intensity of red color was higher in TG CL mice, although the difference was not significant. However, BAT derived from WT CON and CL mice showed no tdTomato fluorescence (Fig. 2G, H).

Browning of inguinal white adipose tissue was induced by aerobic exercise

We analyzed the levels of browning marker genes in iWAT. Exercise significantly increased the levels of UCP1 mRNA expression and other genes including Cidea and PGC-1α (Fig. 3A). It also increased the expression of UCP1 and Cidea protein in iWAT (Fig. 3B). The exercise-induced increase in Cidea expression was also demonstrated by bioluminescence. We obtained *in vivo* images to determine whether browning of the inguinal white adipose tissue was induced by exercise. TG mice subjected to 8 weeks of exercise (Fig. 3D) expressed stronger bioluminescence in iWAT compared with sedentary TG mice (Fig. 3C). Browning was induced by aerobic exercise. Also, TG mice displayed a broader bioluminescence area around inguinal region following exercise (Fig. 3E). In contrast, WT mice showed no bioluminescence regardless of exercise (Fig. 3F). iWAT from TG and WT mice was cryosectioned and imaged to determine tdTomato expression (red) and exercise-induced browning. Lipid droplets of iWAT obtained from exercised TG mice (Fig. 3H) became multilocular compared with sedentary control TG mice (Fig. 3G). Also, the intensity of redness was higher because of increased expression of lipid droplets by tdTomato. After 8 weeks of exercise, iWAT from TG mice turned beige as a result of exercise-induced browning. Because browning was induced by exercise, the lipid droplet

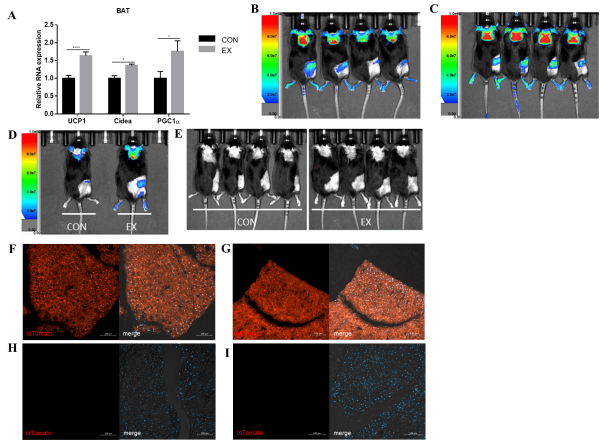

Fig. 1. Exercise-induced activation of BAT. (A) The mRNA expression of brown adipocyte marker genes in BAT. Images of BAT were obtained from (B) TG CON and (C) TG EX. (D) TG EX expressed stronger bioluminescence than TG CON. (E) WT mice showed no bioluminescence. TG and WT mice were injected intraperitoneally with D-luciferin (150 mg/kg body weight) and imaged (blue, green, yellow, and red, ranging from the least to the most intense levels). tdTomato is highly expressed in BAT derived from both (F) TG CON and (G) TG EX. Fluorescence was detected in the whole area. No tdTomato fluorescence was detected in BAT derived from (H) WT CON and (I) WT EX. BAT was cryosectioned and imaged to determine tdTomato fluorescence expression (red). Fluorescent images of tdTomato (left) and combined tdTomato, DAPI, and bright field (right). Sections were mounted in media with DAPI (blue). Scale bars: 100 μm. Sections: 10-20 μm thickness.

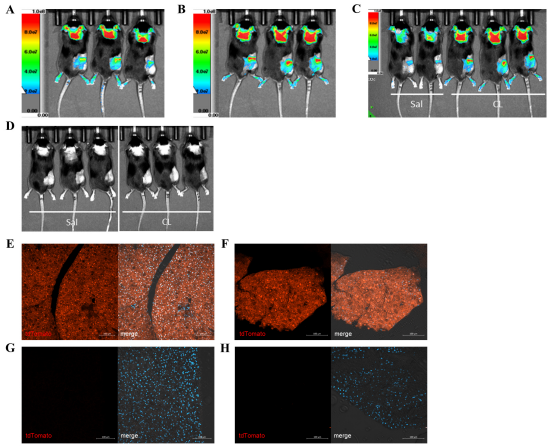

Fig. 2. β3-adrenergic receptor agonist-activated BAT. Images of BAT were obtained from (A) TG CON and (B) TG CL mice. (C) TG CL expressed stronger bioluminescence than TG CON. (D) WT mice did not express bioluminescence. TG and WT mice were injected intraperitoneally with D-luciferin (150 mg/kg body weight) and imaged (blue, green, yellow, and red, ranging from the least to the most intense levels). tdTomato is highly expressed in BAT derived from (E) TG CON and (F) TG CL. Fluorescence was detected in the whole area. No tdTomato fluorescence in BAT derived from (G) WT CON and (H) WT CL. BAT was cryosectioned and imaged to determine tdTomato fluorescence expression (red). Fluorescent images of tdTomato (left) and combined tdTomato, DAPI, and bright field (right). Sections were mounted in media with DAPI (blue). Scale bars: 100 μm. Sections: 10-20 μm thickness.

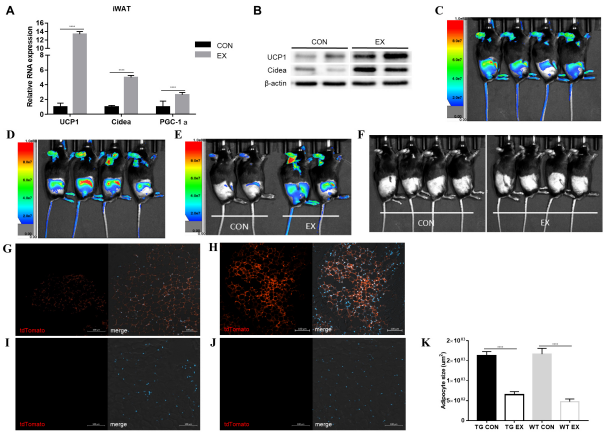

Fig. 3. Exercise-induced browning of iWAT. (A) The mRNA expression of browning marker genes in iWAT. (B) Images of iWAT were obtained from (C) TG CON and (D) TG EX (E) TG EX showed stronger bioluminescence than TG CON. (F) WT mice showed no bioluminescence. TG and WT mice were injected intraperitoneally with D-luciferin (150 mg/kg body weight) and imaged (blue, green, yellow, and red, ranging from the least to the most intense levels). Weak fluorescence was detected in iWAT from (G) TG CON. However, strong fluorescence was found in iWAT from (H) TG EX. No tdTomato fluorescence occurred in iWAT from (I) WT CON and (J) WT EX. iWAT was cryosectioned and imaged to determine tdTomato fluorescence (red). Fluorescent images of tdTomato (left) and combined tdTomato, DAPI, and bright field (right). Sections were mounted in media with DAPI (blue). Scale bars: 100 μm. Sections: 10-20 μm thickness. (K) The area of iWAT adipocytes was measured using ImageJ software (μm²).

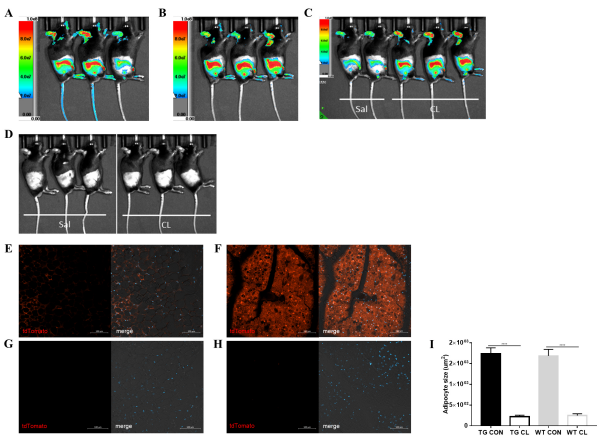

Fig. 4. β3-adrenergic receptor agonist-induced browning of iWAT. Images of iWAT derived from (A) TG CON and (B) TG CL. (C) TG CL expressed stronger bioluminescence than TG CON. (D) WT mouse showed no bioluminescence. TG and WT mouse were injected intraperitoneally with D-luciferin (150 mg/kg body weight) and imaged (blue, green, yellow, and red, ranging from the least to the most intense levels). Weak fluorescence was expressed in iWAT from (E) TG CON. However, strong fluorescence was expressed in iWAT from (F) TG CL. No tdTomato fluorescence was detected in iWAT derived from (G) WT CON and (H) WT CL. iWAT was cryosectioned and imaged to determine tdTomato fluorescence expression (red). Fluorescent images of tdTomato (left) and combined tdTomato, DAPI, and bright field (right). Sections were mounted in media with DAPI (blue). Scale bars: 100 μm. Sections: 10-20 μm thickness. (I) The area of iWAT adipocytes was measured using ImageJ software (μm^2).

size was reduced in exercised WT mice similar to exercised TG mice (Fig. 3J). However, no tdTomato fluorescence was detected similar to sedentary WT mice (Fig. 3I). The area of adipocytes was measured using ImageJ software. Exercise resulted in a remarkable decrease in adipocyte size in iWAT (Fig. 3K).

Browning of inguinal white adipose tissue induced by CL injection

We obtained *in vivo* images to determine if browning of inguinal white adipose tissue was induced by CL. Compared with TG CL mice, TG CON mice (Fig. 4A) expressed weaker bioluminescence in iWAT (Fig. 4B). CL treatment induced browning and increased Cidea expression. Therefore, TG CL mice expressed stronger bioluminescence in iWAT (Fig. 4C). While TG mice expressed bioluminescence in iWAT, WT mice showed no bioluminescence even after browning was induced by CL (Fig 4D). The iWAT derived from TG and WT mice was cryosectioned and imaged to determine tdTomato expression (red) and browning induced by CL treatment. Lipid droplets of iWAT derived from TG CL mice (Fig. 4F) became multilocular compared with those of TG CON mice (Fig. 4E), increasing Cidea-tdTomato expression, suggesting that tdTomato was strongly expressed around multilocular lipid droplets. The lipid droplets became multilocular in iWAT derived from WT CL mice, but showed no tdTomato fluorescence (Fig. 4H). No fluorescence was detected in WT CON mice (Fig. 4G). The adipocyte area was measured using ImageJ software. We found that CL induced a significant decrease in adipocyte size in iWAT (Fig. 4I).

DISCUSSION

Given the current increase in the rates of obesity worldwide, exercise represents an important treatment strategy. It is well known that cold adaptation and β3-adrenergic receptor agonist induce browning. The browning effects and increased energy consumption induced by exercise are not widely known. Exercise is not only the most ethical way to induce browning, but also a therapeutic strategy to ameliorate type 2 diabetes and cardiovascular disease (12, 13).

Reporter genes are used in several research studies because of convenience and lack of toxicity. Many mouse models have been created using reporter genes in connection with imaging technologies (14). They are widely used to trace specific genes and observe changes in their *in vivo* levels (15). Our study confirmed that transgenes work normally in transgenic mice but not in wild-type mice. Also, the changes in Cidea gene expression during browning were identified via luciferase bioluminescence *in vivo* and tdTomato fluorescence in vitro.

BATs of control and EX or CL groups appeared to show similar levels of tdTomato fluorescence. However, *in vivo* images of BATs from EX and CL groups revealed stronger bioluminescence in BAT, which was activated by exercise or CL. The Ami X protocol was used to detect bioluminescence of whole tissues unlike sectioned tissue.

We showed that Cidea gene expression was highly upregulated after 8 weeks of aerobic exercise, especially in iWAT. Also, partial browning was detected via fluorescence in iWAT from the exercised mouse. Exercise induced browning of white adipose tissue.

MATERIALS AND METHODS

Animals

Mice carrying a dual reporter system (Cidea-P2A-Luc2-T2A-tdTomato, Luciferase/tdTomato) were obtained from Young Jae Lee of Gachon University. Mice showed 129 background and hybrid C57BL/6N. Mice were housed in a temperature-controlled room at 22-24°C and a humidity of 50-60% under a 12-h light/dark cycle, with free access to regular chow diet (NIH-31, Ziegler Bros, PA) and water. An equal number of male mice aged 8 weeks were used in the exercise and control groups. They weighed 25 to 30 g each during exercise period. An equal number of 7-week-old male mice were used in CL-316,243 and control groups. All animal experiments were performed according to the "Guide for Animal Experiments" (Edited by the Korean Academy of Medical Sciences) and approved by the Institutional Animal Care and Use Committee (IACUC) at the Seoul National University (Approval Number SNU-200903-1-1).

Exercise

Eight-week-old male mice performed voluntary wheel running for 8 weeks. The running group included 4 Cidea reporter mice and the sedentary control group included 4 wild-type mice. Each mouse was placed in a single housing cage equipped with an active wheel running machine (Activity wheel, TECNIPLAST, Italy) to perform voluntary wheel running for a total distance of 6 to 12 km per day. Similarly, a sedentary control mouse was housed alone in a single cage for the same period without a running wheel.

CL-316,243

Male Cidea reporter mice and wild-type mice aged 7 weeks were used in equal numbers in CL-316,243 (Tocris Bioscience, UK) injection and control groups. Mice were injected with 1 mg of CL-316,243 per kg of body weight intraperitoneally for 3 days. Control mice were injected with saline during the same period.

Real-time PCR analysis

Total RNA from fat tissues was extracted with TRIzol reagent (Life Technologies, USA) according to the manufacturer's standards. First-strand cDNA for PCR analysis was generated with AccuPower® RT PreMix (Bioneer, Korea). Real-time PCR analysis was performed using QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, USA). The qRT-PCR primers are described on supplementary material.

Western blot analysis

Fat tissues were lysed in a RIPA Lysis and Extraction Buffer (Thermo, USA) supplemented with protease and phosphatase inhibitors (genDEPOT, USA). Proteins were separated by SDS-PAGE and transferred to a transfer membrane. Membranes were washed 2 × 10 min with TBST (0.247 M Tris, 27 mM potassium chloride, 1.37 M sodium chloride, 0.5% Tween-20) and blocked with 5% skim milk based on TBST for 1 h at room temperature. After 10 min washing, the membranes were incubated with primary antibodies against UCP1 (ab10983, abcam, 1:1000), Cidea (ab8402, abcam, 1:1000), and β-Actin (A1978, Sigma, 1:10000) at 4°C overnight. Membranes were washed for 1 h, incubated with secondary antibodies (anti-rabbit 1:5,000 and anti-mouse 1:5,000) for 1 h 30 min at room temperature and washed for 6 times for 10 min each time. Each membrane was then placed in a detection solution (Bio-RAD, USA), followed by incubation for 2 min at room temperature and detection by ChemiDoc XRS+ (Bio-RAD, USA).

Luciferin injection

To prepare a stock solution (15 mg/ml), we dissolved 1.0 g of D-Luciferin (PerkinElmer, USA) in 66.6 ml of DPBS (WELGENE, Korea). Mice were injected with 10 μl/g of body weight (150 mg/kg body weight) via intraperitoneal injection. The image was obtained using Ami X (Spectral Instruments Imaging, USA) after 15-20 min of injection. The lateral inguinal white adipose tissue of the mouse was analyzed. Also, the BAT in the interscapular area was analyzed.

Cryosection

We fixed samples in 4% paraformaldehyde (Biosesang, Korea) for 48 h at room temperature. Samples were in 15% sucrose (Sigma-Aldrich, USA) solution for 3 days and 30% sucrose solution for 3 days. After removing the surface sucrose solution with a gauze or non-dust paper, we molded the sample in OCT compound (Sakura Finetek, JAPAN). Tissue sections measuring 10-20 μm each in thickness were obtained using a Cryostat (HM 525, Thermo Fisher Scientific, USA) at −30°C. Images of samples were acquired with a confocal microscope (LSM 800, Zeiss, Germany). The size of adipocytes was measured using the ImageJ software program (version 1.45, National Institutes of Health, USA).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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