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FABP3 and brown adipocyte-characteristic mitochondrial fatty acid oxidation enzymes are induced in beige cells in a different pathway from UCP1

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ABSTRACT

Cold exposure and β_3-adrenergic receptor agonist (CL316,243) treatment induce the production of beige cells, which express brown adipocytes(BA)-specific UCP1 protein, in white adipose tissue (WAT). It remains unclear whether the beige cells, which have different gene expression patterns from BA, express BA-characteristic fatty acid oxidation (FAO) proteins. Here we found that 5 day cold exposure and CL316,243 treatment of WAT, but not CL316,243 treatment of primary adipocytes of C57BL/6J mice, increased mRNA levels of BA-characteristic FAO proteins. These results suggest that BA-characteristic FAO proteins are induced in beige cells in a different pathway from UCP1.

1. Introduction

Two types of adipose tissue are found in mammals: white and brown. White adipose tissue (WAT) is highly adapted to store excess energy in the form of triglycerides. Conversely, brown adipose tissue (BAT) oxidizes chemical energy to produce heat in response to cold exposure. Uncoupling protein-1 (UCP1) and fatty acids play an important role in thermogenesis in BAT. UCP1 is specifically expressed in BAT and is localized to the inner membrane of the mitochondria. Its physiological role is to uncouple oxidative phosphorylation so that most of the energy is dissipated as heat rather than being converted to ATP. Fatty acids also play a key role in thermogenesis as the source of oxidative fuel in the mitochondria.

Compared to white adipocytes (WA), brown adipocytes (BA) contain different types of proteins involved in fatty acid oxidation (FAO). These differences appear to reflect functional differences in the two types of adipose tissue. For example, the expression of fatty acid binding protein 3 (FABP3) is dramatically enhanced in acute cold exposure and is thought to be essential for FAO in BAT; in contrast, FABP3 expression is negligible in WAT [1–3]. FABP3 is a member of the fatty acid binding protein family, which consists of 14–15 kDa intracellular proteins that reversibly bind to hydrophobic ligands, such as saturated and unsaturated long-chain fatty acids, with high affinity. FABPs act as lipid "chaperones" that have been implicated in fatty acid uptake, transport, and targeting [4]. Among these families, FABP3 in BA is thought to transport and deliver fatty acids to the mitochondria for oxidation. Fatty acids delivered to the mitochondria are β-oxidized by mitochondrial enzymes. Proteomic analyses of mitochondria from brown and white adipocytes revealed that their proteomes are considerably different, both qualitatively and quantitatively, and are further characterized by tissue-specific protein isoforms [5]. It has been shown that BA, compared with WA, express characteristic mitochondrial FAO enzyme isoforms such as acyl-Coenzyme A (acyl-CoA) synthetase short-chain family member 1 (ACSS1), acyl-CoA synthetase long-chain family member 5 (ACSL5), carnitine palmitoyltransferase 1b (CPT1b), long-chain acyl-CoA dehydrogenase (ACADL),

Abbreviations: WAT, white adipose tissue; BAT, brown adipose tissue; UCP1, uncoupling protein 1; WA, white adipocyte; BA, brown adipocyte; FAO, fatty acid oxidation; FABP3, fatty acid binding protein3/heart-type fatty acid binding protein; Acyl-CoA, acyl-Coenzyme A; ACSS1, acyl-CoA synthetase short-chain family member 1; ACSL5, acyl-CoA synthetase long-chain family member 5; CPT1b, carnitine palmitoyltransferase 1b; ACADL, long-chain acyl-Coenzyme A dehydrogenase; ACADM, medium-chain acyl-Coenzyme A dehydrogenase; ACADS, short-chain acyl-Coenzyme A dehydrogenase; ACAA2, 3-oxoacyl-Coenzyme A thiolase; β_3AR, β_3-adrenergic receptor; cAMP, cyclic AMP; CL, CL316,243; mRNA, messenger RNA; subWAT, subcutaneous WAT; i.p, intraperitoneal; SV, stromal-vascular; cDNA, complementary DNA; Rps18, ribosomal protein S18; PPAR, peroxisome proliferator-activated receptor; PGC-1, PPARγ coactivator 1.

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medium-chain acyl-CoA dehydrogenase (ACADM), short-chain acyl-CoA dehydrogenase (ACADS), and 3-oxoacyl-CoA thiolase (ACAA2). Defects in the *Acadl* or *Acads* gene of mice resulted in an inability to maintain body temperature under cold conditions [6], which suggests that mitochondrial FAO enzymes play a vital role in thermogenesis. Fatty acids delivered to the mitochondria are activated to fatty acyl-CoAs by acyl-CoA synthetases such as ACSS1 and ACSL5. Once activated, long-chain fatty acids require carnitine palmitoyltransferase, including CPT1b, to be transported into mitochondrial matrix. In the matrix space, acyl-CoA dehydrogenases such as ACADL, ACADM and ACADS, and ACAA2 catabolize acyl-CoAs, which are ultimately processed to produce acetyl-CoAs. Thereafter, acetyl-CoAs enter the citric acid cycle and electron transport chain.

Recently, it has been reported that brown fat-like adipocytes having a multilocular morphology and expressing the BA-specific UCP1 protein exist within certain WATs in mice and rats [7]. These cells have been called recruitable brown fat cells, brown in white (brite) cells, or beige cells [8], and they become more prominent upon prolonged stimulation by cold or β_3-adrenergic receptor (β_3AR) agonists such as CL316,243 (CL) that elevate intracellular cyclic AMP (cAMP) [9]. This brown-like transformation of WAT is the most notable in the inguinal subcutaneous depot [10]. The gene expression pattern and origin of beige cells have been reported to be distinct from those of BA [11,12]. However, it has not been well documented whether BA-characteristic FAO proteins are up-regulated in beige cells.

In this study, using C57BL/6J mice, we demonstrated that cold exposure or β_3AR agonist treatment increased messenger RNA (mRNA) and protein expression of FABP3 and increased mRNA levels of several BA-characteristic mitochondrial FAO enzymes in subcutaneous WAT (subWAT). In addition, using primary adipocytes isolated from subWAT, we examined the effect of a β_3AR agonist or cAMP enhancer on the expression of these proteins in adipocytes. Unexpectedly, our results suggest that these BA-characteristic FAO proteins are induced in a different pathway from UCP1.

2. Materials and methods

2.1. Animals

C57BL/6J mice (4 or 8 weeks old, CLEA Japan, Tokyo, Japan) were fed standard rodent chow pellets and water *ad libitum* and were housed at 23 ± 1 °C on a 12 h light/dark cycle. All experimental procedures were conducted in compliance with protocols approved by the Ethical Committee for the Research of Life Science in Kurume University.

2.2. Cold exposure and β_3-adrenergic receptor agonist (CL316,243) treatment in vivo

Eight-week-old male mice were fed standard rodent chow pellets and water *ad libitum*. The mice were housed individually in plastic cages and divided into two groups that were counterbalanced by body mass. For the cold exposure studies, control groups were maintained at 23 ± 1 °C, whereas cold exposure groups were maintained at 4 °C for 5 days. For the CL treatment studies, control groups were injected intraperitoneally (i.p) once daily with saline (200 μl) for 5 days, whereas CL treatment groups were injected i.p once daily with CL (1 mg/kg; Tocris Bioscience, Bristol, UK) in saline (200 μl) for 5 days. After the mice were killed by decapitation, their posterior subcutaneous fat pads (inguinal-dorsolumbar portion) were separated immediately and used for subsequent processing and analyses.

2.3. Adipose tissue fractionation

Adipose tissue was divided into adipocyte and stromal-vascular (SV) fractions. Freshly excised subcutaneous fat pads from 8-week-old male C57BL/6J mice were rinsed in PBS, minced with scissors, and digested with 3 mg/ml collagenase type II (Worthington, Lakewood, NJ, USA) in isolation buffer (123 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 5 mM glucose, 100 mM HEPES, and 4% BSA, pH 7.4) for 1 h at 37 °C. The digested tissue was filtered through a 200 μm nylon mesh to remove undigested tissue and centrifuged at 210×*g* for 1 min. The mature adipocytes floated to the surface, and the SV cells (capillary, endothelial, mast, macrophage, and epithelial cells) were deposited. The floating cells and the SV cells were washed twice with the isolation buffer, recentrifuged at 210×*g*, and collected as the mature adipocytes and SV cells, respectively. Total RNA from the mature adipocytes and SV cells was isolated using TRIzol (Life Technologies Corporation, Carlsbad, CA) reagent. Adequate separation of adipocytes and SV cells was confirmed by RT-PCR for the adipocyte markers *adiponectin* and *Ucp1* and the SV cell marker *Ucp2* (data not shown).

2.4. Quantitative RT-PCR analysis

Total RNA from mouse tissues or cultured cells was isolated using the TRIzol method combined with RNeasy mini columns (QIAGEN, Valencia, CA) according to the manufacturer's instructions. For quantitative RT-PCR, 0.5–1 μg of total RNA was used to synthesize complementary DNA (cDNA). Target cDNA levels were quantified by real-time PCR by using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) with SYBR Green (Applied Biosystems). Relative mRNA expression levels were calculated using mouse ribosomal protein S18 (*Rps18*). The primer sequences were as follows: *Acaa2* (forward: 5′-ggctctggtttccagtccatc-3′ ; reverse: 5′-gaagcgcacatttctgacacagta-3′), *Acadl* (forward: 5′-ctacctcatgcaagagcttccaca-3′; reverse: 5′-cttcaaacatgaactcacaggcaga-3′), *Acadm* (forward: 5′-tgatgtggcggccattaaga-3′; reverse: 5′-gggttagaacgtgccaacaagaa-3′), *Acads* (forward: 5′-aagtttggatccgcacagcag-3′; reverse: 5′-caagctttggtgccgttgag-3′), *Acsl5* (forward: 5′-cattcggcgggacagtttg-3′; reverse: 5′-atcccattgcagccctgaag-3′), *Acss1* (forward: 5′-agatcctgaagactctgcctgtcc-3′; reverse: 5′-ttgcatcactcaccaatgtcca-3′), *Cpt1b* (forward: 5′-gagacaggacactgtgtgggtga-3′; reverse: 5′-tggtacgagttctcgatggcttc-3′), *Fabp3* (forward: 5′-tggctagcatgaccaagcctactac-3′; reverse: 5′-gttccacttctgcacatggatga-3′), *Rps18* (forward: 5′-ttctggccaacggtctagacaac-3′; reverse: 5′-ccagtggtcttggtgtgctga-3′), and *Ucp1* (forward: 5′-gggcattcagaggcaaatcag-3′; reverse: 5′-ctgccacacctccagtcattaag-3′).

2.5. ELISA

Tissue concentrations of FABP3 protein were measured using a sandwich-type ELISA (Hycult Biotechnology, Uden. The Netherlands) according to the manufacturer's protocol. The values were normalized to the total protein concentrations determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

2.6. Primary cell culture and treatment

For the culture of primary subcutaneous white adipocytes, posterior subcutaneous fat pads (inguinal-dorsolumbar portion) were isolated from 4-week-old C57BL/6J male mice. The isolated tissues were rinsed in PBS, minced with scissors, and digested with 1 mg/ml collagenase type II (Worthington) in isolation buffer at 37 °C for 30 min. Cell suspensions were filtered through a 100 μm filter and centrifuged at 210×*g* for 10 min. The pellet consisting of preadipocytes was resuspended in 1 mL of red blood cell lysis buffer (IBL, Gunma, Japan). After incubation for 3 min at

room temperature, 10 ml plating medium (Dulbecco's modified Eagle's medium supplemented with ascorbate, biotin, pantothenate, triiodothyronine, octanoic acid, penicillin–streptomycin, and FCS from a white adipocyte culture kit [TaKaRa Bio, Shiga, Japan]) was added to the cell suspension. To remove endothelial cell clumps, the cell suspension was filtered through a sterile 20 μm mesh filter. Preadipocytes were recovered by centrifugation and washed in plating medium twice. The preadipocytes were then plated on 12-well tissue culture plates at a density of 8×10^4 cells/cm^2 in plating medium and cultured at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. The medium was changed on day 1 and then every second day. Induction of differentiation was performed using differentiation medium (plating medium supplemented with insulin, dexamethasone, and 3-isobutyl-L-methylxanthin [TaKaRa Bio]) for 2 days. Subsequently, cells were cultured in maintenance medium (plating medium supplemented with insulin [TaKaRa Bio]) for 8 days. The cells were incubated in serum-free medium for 12 h prior to harvest of cultures, and the cultures were treated with or without 1 μM CL or 10 μM forskolin for 6 h prior to harvesting.

2.7. Statistical analysis

All experimental data are presented as the mean ± SE. Comparisons were performed by two-tailed *t*-tests. The criterion for statistical significance was $p < 0.05$ for all tests (Graph Pad PRISM 5.0a; Graph Pad Software Inc., La Jolla, CA).

3. Results

3.1. Cold exposure and β_3-adrenergic receptor agonist (CL316,243) treatment induced gene expression of Fabp3 and BA-characteristic mitochondrial fatty acid oxidation enzymes in subcutaneous WAT in vivo

To confirm the browning of WAT after 5-day cold exposure (4 °C) or CL treatment, we studied *Ucp1* mRNA expression in subWAT by using quantitative RT-PCR. Both 5-day cold exposure and CL treatment significantly induced *Ucp1* mRNA expression (Fig. 1A). As expected, brown-like transformation of subWAT occurred after both 5-day cold exposure and CL treatment.

FABP3 is essential for fatty acid oxidation in BAT, whereas its expression is negligible in WAT [1–3]. To examine the effects of cold exposure or CL treatment on FABP3 expression, we investigated *Fabp3* mRNA expression in subWAT. Cold exposure and CL treatment markedly increased *Fabp3* mRNA expression by 102-fold and 22-fold, respectively compared to the control ($p < 0.01$, Fig. 1B).

WAT is composed of mature adipocytes and the SV fraction. To determine which cell populations in subWAT express FABP3, we measured *Fabp3* mRNA levels in fractionated adipose tissue. Fractionation of WAT by centrifugation showed that *Fabp3* was mainly expressed in mature white adipocytes rather than in the SV fraction, after both cold exposure and CL treatment (Fig. 1B).

Furthermore, we analyzed the content of protein by using a FABP3 immunoassay, which showed that cold exposure and CL

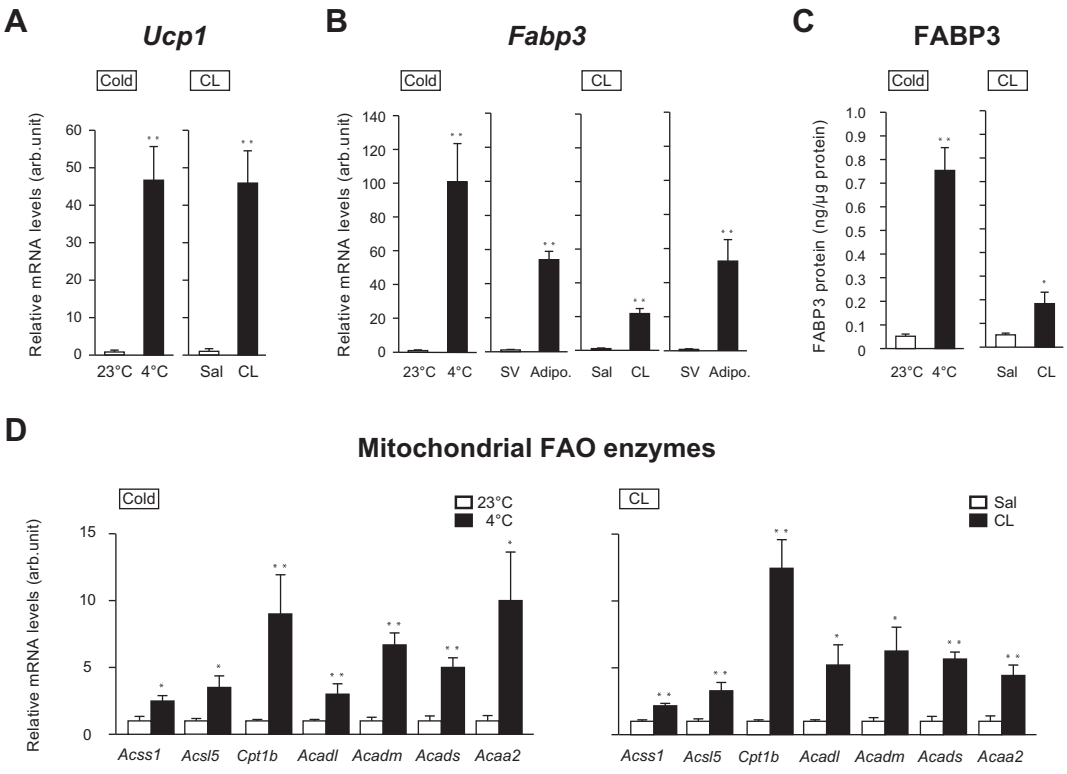

Fig. 1. Cold exposure and β_3-adrenergic receptor agonist (CL316,243) treatment induced gene expression of *Fabp3* and BA-characteristic mitochondrial FAO enzymes in subcutaneous white adipose tissue. C57BL/6J mice at 8 weeks of age maintained at 23 °C or 4 °C for 5 days. (A–D, left panels) C57BL/6J mice at 8 weeks of age were injected i.p once daily with saline (Sal) or CL316,243 in saline (CL) for 5 days. (A–D, right panels) The subcutaneous fat pads of the mice were used for the following analyses. (A) *Ucp1* mRNA levels in subcutaneous white adipose tissues (subWAT). (B) *Fabp3* mRNA levels in subWAT and the adipocyte fraction (Adipo.) compared to the stromal-vascular fraction (SV) of subWAT. (C) FABP3 protein concentrations in subWAT determined by an immunoassay. Values are expressed as nanograms of FABP3 per microgram of total cellular protein. (D) mRNA levels of BA-characteristic mitochondrial fatty acid oxidation (FAO) enzymes (*Acss1*, *Acsl5*, *Cpt1b*, *Acadl*, *Acadm*, *Acads*, and *Acaa2*) in subWAT. Gene expression was determined by real-time RT-PCR and normalized against 18S rRNA levels. In all panels, values represent the means ± SE ($n = 4–5$). $^*p < 0.05$, $^{**}p < 0.01$.

treatment increased the content of FABP3 protein by 14-fold and 3-fold, respectively, compared to the control ($p < 0.01$ and $p < 0.05$, respectively, Fig. 1C). Thus, FABP3 expression increases with the brown-like transformation of WAT.

The increased FABP expression in WAT implies that more fatty acids are transported to the mitochondria for oxidation. Fatty acids delivered to the mitochondria are β-oxidized by mitochondrial enzymes. We therefore investigated whether cold exposure or CL treatment increases the levels of BA-characteristic mitochondrial FAO enzymes in subWAT, and found that the mRNA levels of *Acss1*, *Acsl5*, *Cpt1b*, *Acadl*, *Acadm*, *Acads*, and *Acaa2* increased under both conditions (Fig. 1D).

3.2. CL316,243 or cAMP enhancer (forskolin) treatment did not induce the expression of Fabp3 or BA-characteristic mitochondrial FAO enzymes in primary adipocytes of subWAT

To investigate the direct effect of CL or a cAMP enhancer, forskolin, on the adipocytes of subWAT, we used a culture of primary adipocytes isolated from subWAT depots. CL or forskolin treatment for 6 h increased *Ucp1* mRNA expression by 29-fold and 39-fold, respectively, compared to the control ($p < 0.01$, Fig. 2A). These results suggest that *Ucp1* expression is induced by the β_3AR-cAMP pathway and that the primary adipocytes are beige cells.

It was previously reported that norepinephrine elevated the transcript level of FABP3 in primary brown adipocytes and a brown adipocyte cell line [1,2]. However, in beige cells, the effects of β_3AR agonists on the transcript level of FABP3 are not clear. To study whether FABP3 is induced by the β_3AR-cAMP pathway, we examined *Fabp3* mRNA expression following CL or forskolin treatment. CL or forskolin treatment for 6 h decreased the level of *Fabp3* mRNA (Fig. 2B).

Furthermore, we investigated the effect of CL or forskolin treatment on the mRNA levels of BA-characteristic mitochondrial FAO enzymes. CL or forskolin treatment for 6 h did not induce but rather reduced the mRNA levels of *Acss1*, *Acsl5*, *Cpt1b*, *Acadl*, *Acadm*, *Acads*, and *Acaa2* (Fig. 2C).

4. Discussion

It has been reported that beige cells that express UCP1 protein become more prominent within WAT in mice after cold exposure or β_3AR agonist treatment. The objective of this study was to determine whether FABP3 and BA-characteristic mitochondrial FAO enzymes are induced after brown-like transformation of WAT. We present evidence here that cold exposure or CL treatment increases mRNA and protein expression of FABP3 and increases mRNA levels of BA-characteristic mitochondrial FAO enzymes (ACSS1, ACSL5, CPT1b, ACADL, ACADM, ACADS, and ACAA2) in subWAT *in vivo*. In addition, using primary adipocytes, we showed that CL or forskolin treatment increased *Ucp1* mRNA expression; however, it did not change or decrease the mRNA expression of *Fabp3* and BA-characteristic mitochondrial FAO enzymes.

In BAT, the transcript levels of *Fabp3* and *Cpt1b* but not *Acadm* and *Acadl* are elevated by cold exposure [13]. These results suggest that regulation of *Fabp3* and *Cpt1b* expression is the rate-limiting step in FAO, and the constitutive expression of *Acadm* and *Acadl* is sufficient to enhance β-oxidation in BAT. Some reports have mentioned that the gene expression of *Cpt1b* is increased in WAT after cold exposure or CL treatment [14]. In this study, we found that the gene expression of not only *Cpt1b* but also *Fabp3*, *Acadm*, and *Acadl* was induced by cold exposure in subWAT. The constitutive expression of *Fabp3*, *Acadm*, and *Acadl* is lower in WAT than in BAT; therefore, the up-regulation of these genes is considered to be necessary to enhance β-oxidation with brown-like transformation

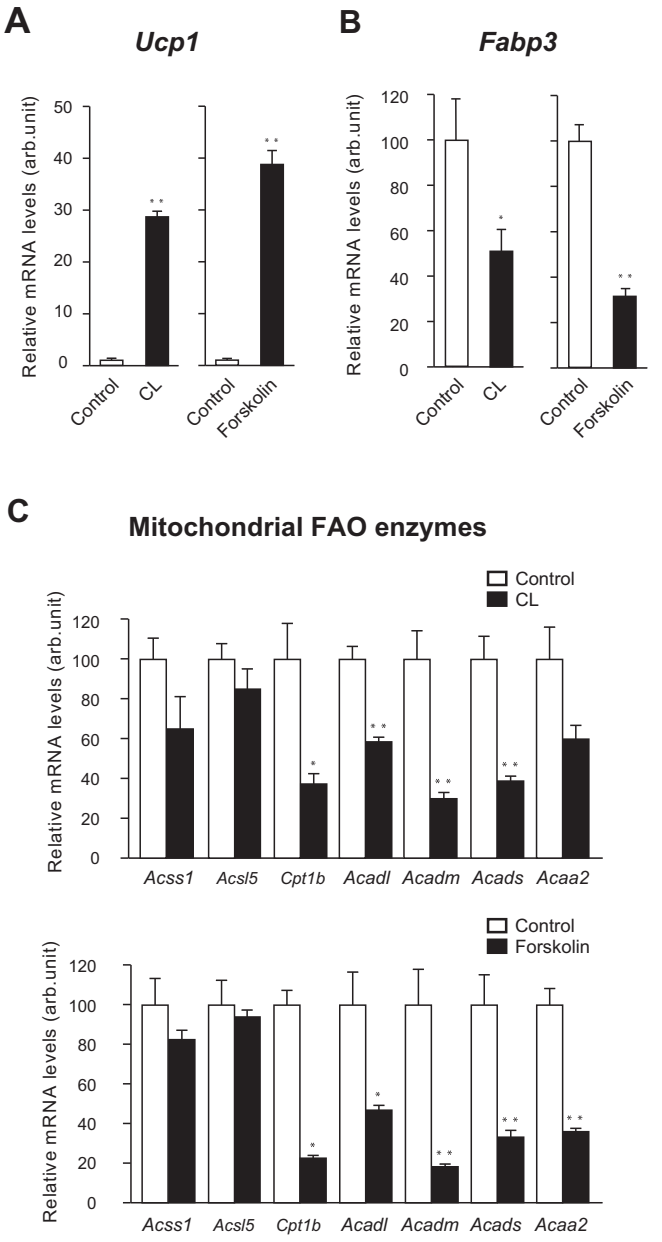

Fig. 2. β_3-adrenergic receptor agonist (CL316,243) or cAMP enhancer (forskolin) treatment did not induce gene expression of *Fabp3* and BA-characteristic mitochondrial FAO enzymes in primary adipocytes of subWAT. Primary adipocytes of subWAT were grown as described in Section 2 and used for the following analyses. Cells were pretreated for 6 h with or without 1 μM CL316,243 (CL) or 10 μM forskolin. Gene expression of *Ucp1* (A), *Fabp3* (B), and BA-characteristic mitochondrial FAO enzymes (*Acss1*, *Acsl5*, *Cpt1b*, *Acadl*, *Acadm*, *Acads*, and *Acaa2*) (C) was determined by real-time RT-PCR and normalized against 18S rRNA levels. In all panels, values represent the means ± SE ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$.

in WAT. Our findings suggest that BA-characteristic FAO proteins are inducible, and the increased levels of the proteins appear to reflect enhanced β-oxidation with brown-like transformation of WAT.

β_3AR is of pivotal importance in the brown-like transformation of WAT after cold exposure because β_3AR knockout mice show decreased occurrence of the brown-like transformation of WAT [15]. We showed that CL treatment increased the levels of FABP3 and BA-characteristic mitochondrial FAO enzymes in subWAT. By contrast, using primary adipocytes, we found that CL and forskolin treatment increased *Ucp1* mRNA expression but did not change or decrease the mRNA expression of *Fabp3* or BA-characteristic

mitochondrial FAO enzymes. Our culture experiments suggest that FABP3 and BAT-characteristic mitochondrial FAO enzymes are induced in a different pathway from UCP1 induction in beige cells.

Many genes involved in FAO have been shown to be induced by peroxisome proliferator-activated receptor α (PPARα) [16]. PPARα is a fatty acid-activated nuclear receptor and known to be an important regulator of mitochondrial β-oxidation in tissues such as the heart, liver, and BAT. However, the expression of PPARα in WAT is very low and its function remains unclear. It has been reported that ectopic overexpression of PPARα and PPARγ coactivator 1 (PGC-1) in adipocyte-like differentiated 3T3L-1 cells cooperatively induces the expression of mitochondrial FAO enzyme genes including *Acadm* and *Acadl* [17]. PGC-1 serves as a transcriptional coactivator in the control of mitochondrial FAO enzyme gene expression. We confirm that the *Ppara* mRNA level increases in subWAT after CL treatment *in vivo* as reported previously by Li et al. [18]. However, we found that the *Ppara* mRNA level was decreased in the primary adipocytes after CL treatment (data not shown). PPARα could be essential for the expression of several BAT characteristic proteins involved in FAO in subWAT. Although FABP3 is reported to augment the transcriptional activity of PPARα [19], the regulatory mechanism of *Ppara* expression in WAT remains unclear. Further studies are needed to uncover the molecular mechanism for the regulation and function of PPARα in WAT.

A limitation of our study is the heterogeneity of the primary adipocytes isolated from subWAT, as cellular heterogeneity was previously shown for clonal cells derived from the subWAT of mice [12]. Therefore, more precise isolation and purification of beige cells in subWAT are required for further investigation.

Elucidating the molecular mechanism involved in FAO in beige cells has important medical implications and may provide clues in the development of anti-obesity agents. Recent studies have shown that thermogenic UCP1-positive adipocytes exist in adult humans, and the most of these adipocytes in humans are molecularly similar to murine beige cells rather than brown adipocytes [12,20]. Modulation of the expression of BA-characteristic FAO proteins in beige cells may represent a novel therapeutic target for obesity and metabolic diseases.

In summary, we show that the expression of BA-characteristic FAO proteins is increased in subWAT after cold exposure or CL treatment. Moreover, primary culture experiments suggest that these proteins are induced in a different pathway from UCP1. Further investigation is necessary to determine the regulatory mechanism for the expression of BA-characteristic FAO proteins in subWAT.

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