Original Article

Acetate alters expression of genes involved in beige adipogenesis in 3T3-L1 cells and obese KK-Ay mice

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The induction of beige adipogenesis within white adipose tissue, known as "browning", has received attention as a novel potential anti-obesity strategy. The expression of some characteristic genes including *PR domain containing 16* is induced during the browning process. Although acetate has been reported to suppress weight gain in both rodents and humans, its potential effects on beige adipogenesis in white adipose tissue have not been fully characterized. We examined the effects of acetate treatment on 3T3-L1 cells and in obese diabetic KK-Ay mice. The mRNA expression levels of genes involved in beige adipocyte differentiation and genes selectively expressed in beige adipocytes were significantly elevated in both 3T3-L1 cells incubated with 1.0 mM acetate and the visceral white adipose tissue from mice treated with 0.6% acetate for 16 weeks. In KK-Ay mice, acetate reduced the food efficiency ratio and increased the whole-body oxygen consumption rate. Additionally, reduction of adipocyte size and *uncoupling protein 1*-positive adipocytes and interstitial areas with multilocular adipocytes appeared in the visceral white adipose tissue of acetate-treated mice, suggesting that acetate induced initial changes of "browning". In conclusion, acetate alters the expression of genes involved in beige adipogenesis and might represent a potential therapeutic agent to combat obesity.

***Key Words*:** **acetate, beige adipogenesis, obesity, visceral fat, short chain fatty acid**

Obesity is one of the most serious health issues worldwide, and effective treatments for this condition are critically needed. As a novel strategy to suppress weight gain, increasing beige adipocytes in white adipose tissue (WAT), known as "browning", has received much recent attention. Brown adipose tissue (BAT) is a specialized type of tissue that dissipates chemical energy by burning lipids to produce heat, which results in increased energy expenditure. Previous studies have shown that the activity of BAT can markedly affect body weight (BW).[1–3] It is now recognized that WAT, which was previously considered to be a specialized tissue for triglyceride storage, can be converted into a "brown-like" state by prolonged cold exposure or the administration of either β-adrenergic compounds or certain secreted factors.[4–6] These "brown-like" adipocytes have been termed beige adipocytes that can be defined by a multilocular lipid droplet morphology and high mitochondrial content. Beige adipocytes express a core set of brown fat genes, such as *peroxisome proliferator-activated receptor* (*PPAR*)-γ *co-activator 1α* (*PGC1α*), *PR domain containing 16* (*PRDM16*), *uncoupling protein 1* (*UCP1*), and *PPARα*, as well as beige adipocyte-selective genes, such as *transmembrane protein 26* (*TMEM26*) and *T-box 1* (*TBX1*).[4–6] As beige adipocytes in WAT can dissipate chemical energy similarly to BAT, increased activity of beige adipocytes can guard against obesity in several animal models.[6–10] Thus, the induction of "browning" represents an attractive potential anti-obesity strategy.

Short chain fatty acids (SCFAs) are used as sources of energy and substrates for the *de novo* synthesis of lipids and glucose, but can also act as signaling molecules.[11] Among SCFAs, acetate represents the most abundant end product generated by the fermentation of undigested carbohydrates in the colon and can also be detected in systemic circulation. Several reports have demonstrated the anti-obesity and anti-diabetic activities of acetate.[12–16] Various molecular mechanisms underlying the beneficial effects of acetate have been reported, including the activation of AMP-activated protein kinase (AMPK) in liver,[14,15,17] increasing glucagon-like peptide-1 (GLP-1) secretion from the intestine,[18] and signaling via specific receptors for SCFAs—the G protein coupled receptors (GPCRs) GPR41 and GPR43.[19–21] However, whether acetate can induce the "browning" of WAT has not yet been determined.

The present study investigated whether acetate could alter the expression of genes involved in beige adipogenesis in WAT and assessed whether acetate-dependent changes in gene expression were associated with enhanced energy expenditure and fat reduction *in vivo*.

Materials and Methods

Cell culture and treatments. Mouse 3T3-L1 pre-adipocytes were cultured in DMEM containing 25 mM glucose with 10% fetal calf serum and 1% antibiotic/antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan) at 37°C and 5% CO_2. At 2 days after confluence (day 0), cell differentiation was initiated by adding 1 μM dexamethasone, 500 μM isobutylmethylxanthine (IBMX), and 0.86 μM insulin. After 3 days (day 3), medium was replaced with culture medium containing 0.86 μM insulin. After 3 days (day 6), medium was replaced with fresh culture medium without insulin.[22,23]

Sodium acetate (acetate; in the form of neutralized acetic acid, 3.50 kcal/g) was purchased from Nacalai Tesque. To investigate the effects of acetate, acetate was added to the culture medium at day 0 and treatment with acetate was continued until cells were harvested. Kimura *et al.*[20] previously treated 3T3-L1 cells with 10 mM acetate, and did not observe cytotoxicity. In this study, we confirmed by microscopic observation that no obvious changes

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Table 1. Primer list

Target gene	Forward	Reverse
ADAM8	aaggtgtctgccctgtgttc	aacttggagccgatgctttc
CIDEA	aggaatctgctgaggtttatgtcc	gggatggctgctcttctgtatc
Dio2	gcgaattgatccaaactggag	agagcacacgcaggacagaa
FABP3	agtcactggtgacgctggacg	aggcagcatggtgctgagctg
PGC1α	atgtgtcgccttcttgctct	atctactgcctggggacctt
PPARα	cctcttcccaaagctccttca	cgtcggactcggtcttcttg
PPARγ	cgaagaaccatccgattgaag	tgagacatccccacagcaag
PRDM16	agagacaaaggcaaggacaagg	gtggcgggaagaaggaatg
TACE	gtacgtcgatgcagagcaaa	aaaccagaacagacccaacg
TBX1	ggcaggcagacgaatgttc	ttgtcatctacgggcacaaag
TMEM26	ctgtgttctcattctcggctttg	gtgcttggtggctcattcttc
UCP1	agctgatgaagtccagacagacag	tggttggttttattcgtggtctc
18s	agtccctgccctttgtacaca	cgatccgagctcacta

in the pattern of differentiation or cell number occurred following acetate treatment.

***In vivo* acetate administration.** Five-week-old male KK-Ay mice were purchased from CLEA Japan (Tokyo, Japan). Mice were housed individually under specific pathogen-free conditions with alternating 12-h periods of light and dark in a temperature controlled room (22 ± 2°C) and were allowed *ad libitum* access to water and standard chow (3.45 kcal/g, 4.6% fat, 51.0% carbohydrate, 24.9% protein by calories; CLEA Japan). After 2 weeks adaptation, mice were divided into two experimental groups: control (Cont) and acetate (Ac) groups. Mice in the Ac group were treated with 0.6% acetate in drinking water for 16 weeks. The food efficiency ratio (BW gain-to-caloric intake ratio) was calculated based on increments of BW and caloric intake for the initial 12 weeks as follows: [BW gain (g/day)/caloric intake (kcal/day) × 100].[24] Because acetate is also an additional source of energy,[25,26] caloric intake could be determined based on the amounts of both food intake and acetate consumed. After 16 weeks of treatment, tissues were isolated under conditions of overnight chow and acetate deprivation to adjust the exogenous energy supply. For tissue isolation, all mice were anesthetized using isoflurane and pentobarbital. Isolated tissues were immediately frozen in liquid nitrogen and stored at −80°C. A portion of adipose tissue was used to prepare paraffin sections, which were subjected to histological analysis. All animal care and experimental procedures were approved by the Animal Care and Use Committee at Kumamoto University (permit number: B27-185).

Measurements of plasma acetic acid concentrations. After 8 weeks of treatment, plasma was obtained between 16:00 to 18:00 while mice were allowed *ad libitum* access to food and drinking water (which contained acetate for the Ac group). Plasma acetate concentrations were measured using a F-Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Indirect calorimetry study. Consumption of oxygen and the amount of activity were determined after 14 weeks of treatment. Mice were individually placed in air-tight cages, and oxygen consumption rate was measured using a metabolic chamber (Model MK-5000RQ/MS, Muromachi Kikai Co., Tokyo, Japan). The amount of activity was measured using an infrared-ray passive sensor system (SUPERMEX, Muromachi Kikai Co.). Mice were housed with alternating 12-h periods of light and dark and were allowed *ad libitum* access to food and drinking water (which contained acetate for the Ac group). Mice were acclimated to the monitoring for more than 24-h before undergoing a 24-h recording period.

Histology. Dissected tissues were fixed in 10% formalin and were paraffin-embedded. Paraffin-embedded WAT was sectioned and stained with hematoxylin and eosin (H&E) using standard techniques. We determined adipocyte sizes from five sections per mouse using a BZ-9000 All-in-One Fluorescence Microscope (Keyence, Osaka, Japan). For immunohistochemistry analysis, paraffin-embedded dewaxed sections were incubated for 2-h with an anti-UCP1 antibody (ab23841, Abcam, Cambridge, UK) at a dilution of 1:200. Secondary detection was performed for 30 min using Histofine Simple Stain Mouse MAX PO (R) (414341, Nichirei, Tokyo, Japan), and sections were counterstained with Mayer's hematoxylin solution to highlight tissue architecture and nuclear location.

RNA isolation and real-time quantitative reverse transcription PCR. The real-time quantitative reverse transcription PCR (qRT-PCR) analysis was performed as reported previously.[27,28] In brief, total RNA from cultured cells and tissues was extracted using Sepasol-RNA I Super G (Nacalai Tesque), and complementary DNAs were synthesized using ReverTraAce qPCR RT Master Mix (Toyobo, Osaka, Japan). The qRT-PCR analysis was performed using Light Cycler Fast-Start DNA Master Plus SYBER Green (Roche, Basel, Switzerland) according to the manufacturer's instructions. Quantitative measurements of mRNA expression were normalized to the levels of 18S ribosomal RNA (18S). Primer sets for qRT-PCR are listed in Table 1.

Data analysis. All data are presented as means ± SD. Comparisons of two groups were conducted using Welch's *t* test. Comparisons for more than three groups were adequately analyzed by one-way ANOVA or two-way repeated measure ANOVA. P values<0.05 were considered to indicate statistically significant differences.

Results

Acetate alters the expression of genes involved in beige adipogenesis in 3T3-L1 cells. To investigate whether acetate could alter the expression of genes involved in beige adipogenesis *in vitro*, 3T3-L1 pre-adipocytes were differentiated into adipocytes in the absence or presence of different concentrations of acetate (0.2, 0.5, and 1.0 mM) for 6 days. As shown in Fig. 1, 1.0 mM acetate significantly increased the mRNA expression levels of *UCP1*, *PRDM16*, *PPARα*, *iodothyronine deiodinase 2* (*Dio2*) and *cell death-inducing DNA fragmentation factor-α-like effector A* (*CIDEA*). Therefore, we decided to use 1.0 mM acetate in the following experiments.

To investigate the time course of acetate treatment-dependent effects, we incubated pre-adipocytes without or with acetate for 3, 6, and 10 days. Incubation with acetate for 3 days significantly increased the mRNA expression levels of *PGC1α*, *UCP1*, *PRDM16*, and *PPARα*, which have been linked to beige adipocyte

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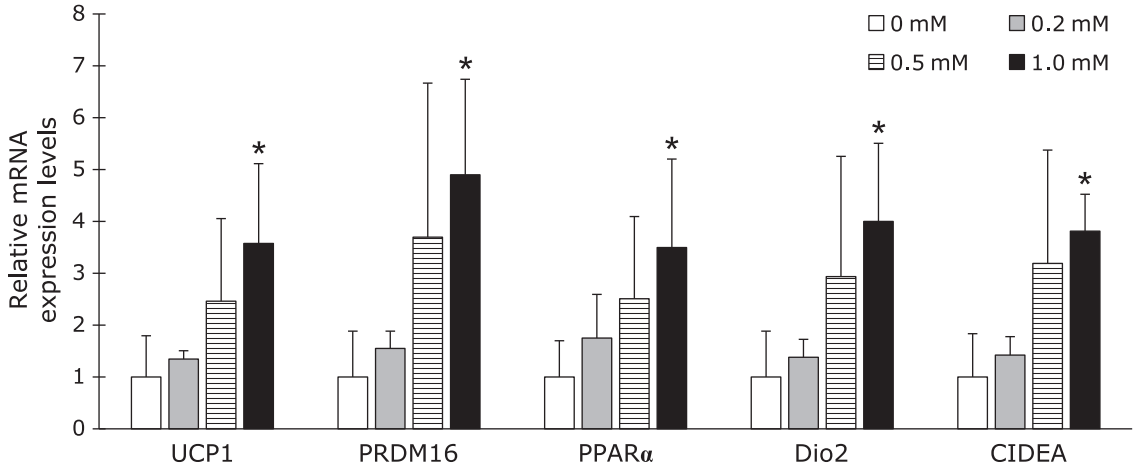

Fig. 1. Effects of acetate on the expression of genes involved in beige adipogenesis in 3T3-L1 cells. 3T3-L1 pre-adipocytes were differentiated for 6 days in the absence (Cont; control) or presence of 0.2, 0.5, or 1.0 mM acetate. Relative mRNA expression levels of the indicated genes measured by qRT-PCR are shown (n = 4–6 per group). Data are presented as means ± SD. Differences between means were analyzed using one-way ANOVA with Bonferroni post-hoc test. *p<0.05 vs control group. Cont, control group.

differentiation and are known brown fat genes. Moreover, acetate also increased mRNA expression levels of the brown fat genes *CIDEA* and *fatty acid binding protein 3* (*FABP3*), as well as the beige adipocyte-selective genes *TMEM26* and *TBX1* (Fig. 2A). At days 6 and 10, the mRNA expression levels of these genes were also continuously upregulated in response to acetate treatment (Fig. 2B and C). These findings strongly suggest that acetate could directly act on pre-adipocytes and induce the expression of genes involved in beige adipogenesis in 3T3-L1 cells.

Additionally, we also examined whether butyrate, which is a type of SCFA, could alter the expression of genes involved in beige adipogenesis. 3T3-L1 pre-adipocytes were differentiated into adipocytes in the absence or presence of butyrate (0.5 mM) for 6 days. The mRNA expression levels of genes which were upregulated in acetate-treated 3T3-L1 cells, such as *PGC1α*, *UCP1*, *PRDM16* and *TMEM26*, were also significantly elevated in butyrate-treated 3T3-L1 cells (data not shown).

Acetate alters the expression of genes involved in beige adipogenesis in epididymal adipose tissue of KK-Ay mice. To test whether the oral administration of acetate could affect the expression of genes involved in beige adipogenesis *in vivo*, KK-Ay mice were treated without or with acetate in drinking water for 16 weeks. The mRNA expression levels of all genes upregulated in acetate-treated 3T3-L1 cells were also significantly elevated in epididymal WAT from acetate-treated mice (Fig. 3A).

Next we examined the effects of acetate on inguinal adipose tissue, a type of subcutaneous WAT, and intrascapular BAT. In subcutaneous WAT, acetate treatment had slight effects on mRNA expression levels of the indicated genes, although the differences were not significant (Fig. 3B). In BAT, the mRNA expression levels of the indicated genes were comparable between the control and acetate treatment groups (Fig. 3C).

We also investigated whether acetate affected the expression of genes involved in both inflammation and tissue remodeling. The mRNA expression levels of a disintegrin and metalloprotease (*ADAM*) 8 and *ADAM17* in epididymal WAT were not affected by acetate treatment (data not shown).

These data suggest that acetate can induce the expression of several key molecules involved in "browning" mainly in visceral WAT.

Acetate reduces the food efficiency ratio and adipocyte size and elevates oxygen consumption in KK-Ay mice. We next tested whether oral administration of acetate could affect BW gain and energy expenditure in acetate-treated KK-Ay mice. As expected, plasma acetate concentrations were 1.9-fold higher in the Ac group compared with the Cont group (Fig. 4A), indicating that acetate reached various organs, including the visceral WAT, at sufficiently high concentrations in acetate-treated mice. Significant differences were not observed in both BW and food intake between the two groups (Fig. 4B and C). The average intake of drinking water was 20.3 g/day, and the average acetate intake was 0.12 g/day in the Ac group. Drinking water intake was significantly elevated in the Ac group (Fig. 4D). BW gain tended to be lower in the Ac group, although the differences were not significant (Fig. 4E). The food efficiency ratio was significantly lower in the Ac group (Fig. 4F), suggesting that oral administration of acetate could suppress BW gain per caloric intake in KK-Ay mice. Additionally, the oxygen consumption rate was significantly elevated in the Ac group (Fig. 4G), while spontaneous physical activity was comparable between the two groups (Fig. 4H).

Finally, we examined histological change and UCP1 expression in visceral WAT from acetate-treated mice. Histological analyses of epididymal WAT revealed that adipocyte size was significantly smaller in the Ac group compared with the Cont group (Fig. 5A). In inguinal WAT, the size of adipocytes was comparable between the two groups (Fig. 5B). Immunohistochemical staining of epididymal WAT from the Ac group revealed the appearance of UCP1-positive adipocytes and interstitial areas containing multilocular adipocytes (Fig. 5C). UCP1 expression was scarcely detected in epididymal WAT from the Cont group.

These findings suggest that the administration of acetate may reduce food efficiency ratio and adipocyte size in visceral WAT by enhancing energy expenditure in this hyperphagic model of obesity.

Discussion

In the present study, the mRNA expression levels of both brown fat and beige adipocyte-selective genes were significantly upregulated by acetate treatment *in vivo* and *in vitro*. Acetate-treated KK-Ay mice showed not only reduced BW gain per caloric intake but also elevated whole-body oxygen consumption, similar to previous reports.[13,29] Based on our results and these previous findings, it is likely that acetate exerts anti-obesity effects, at least in part, through the dissipation of excess energy.

Several studies have reported that acetate can suppress appetite

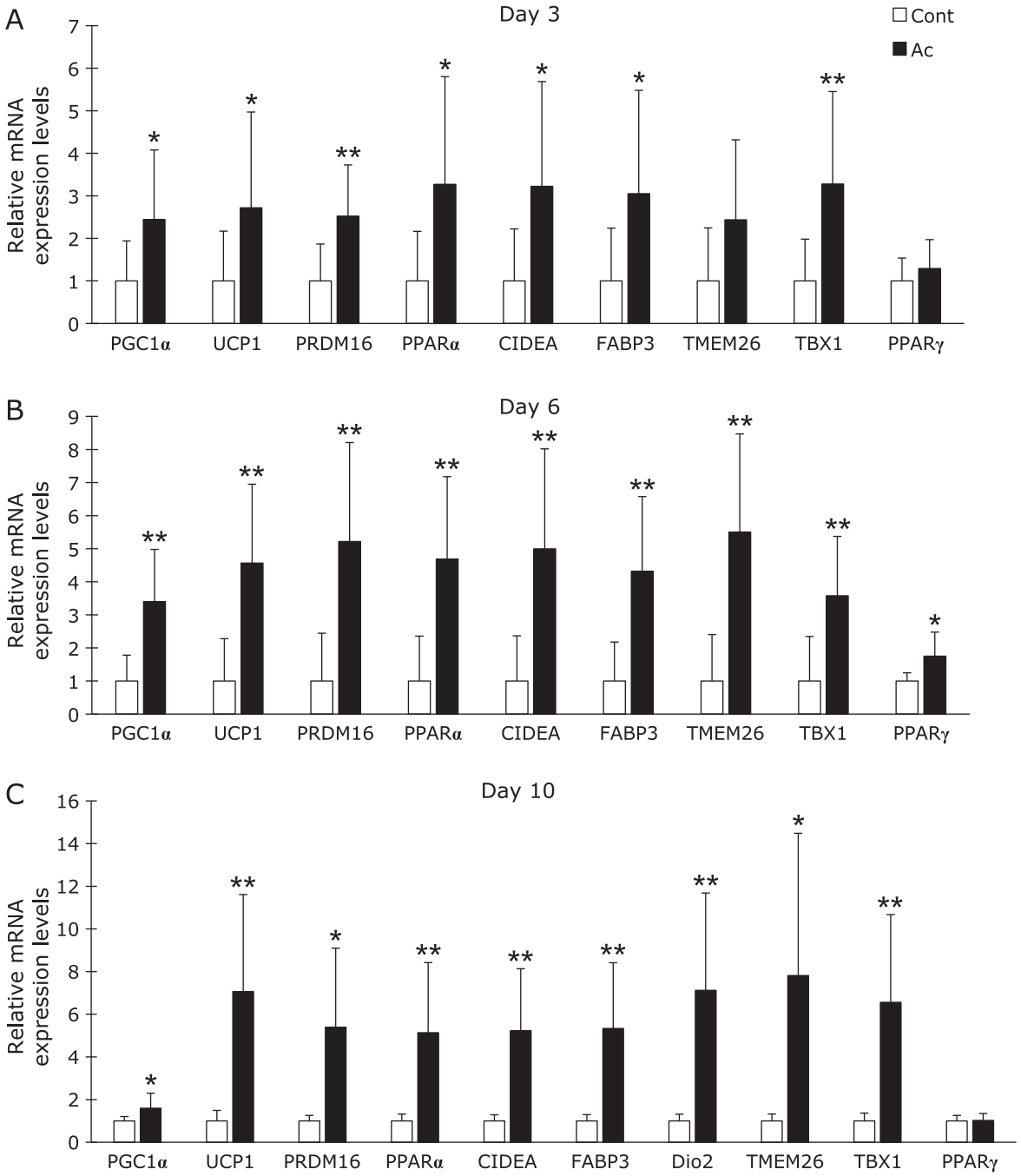

Fig. 2. Time course of acetate-induced effects on gene expression in 3T3-L1 cells. 3T3-L1 pre-adipocytes were differentiated in the absence or presence of 1.0 mM acetate for (A) 3 days (n = 10–11 per group), (B) 6 days (n = 8–9 per group), or (C) 10 days (n = 6–9 per group). The relative mRNA expression levels of the indicated genes measured using qRT-PCR are shown. Differences between the means of two groups were analyzed using Welch's t test. *p<0.05; **p<0.01 vs control group. Cont, control group; Ac, acetate group.

by directly altering hypothalamic neuronal activity or via GLP-1 secretion.[30,31] Moreover, acetate can reduce BW in both rodents and humans.[13,15,16,30,31] In the present study, no significant reductions in food intake or BW were observed in acetate-treated KK-Ay mice, although the food efficiency ratio was significantly reduced. One reason that acetate did not reduce food intake or BW in our study may be differences in the strains of mice used in our study compared with other reports.[13,15,16,30,31] These studies investigated obese models without abnormalities in appetite regulation, such as diet-induced obesity mice, and found that acetate reduced both food intake and BW. On the other hand, a previous study using KK-Ay mice showed no reduction in food intake or BW after acetate administration.[14] Therefore, the dysregulation of appetite induced by Ay gene transfer may explain why acetate-mediated signals did not suppress appetite and BW gain in KK-Ay mice. Future studies that use other animal models without abnormalities in appetite regulation, such as diet-induced obesity, will provide more information about the regulation of appetite and BW by acetate. Meanwhile, our present study detected significantly elevated drinking water intake in the Ac

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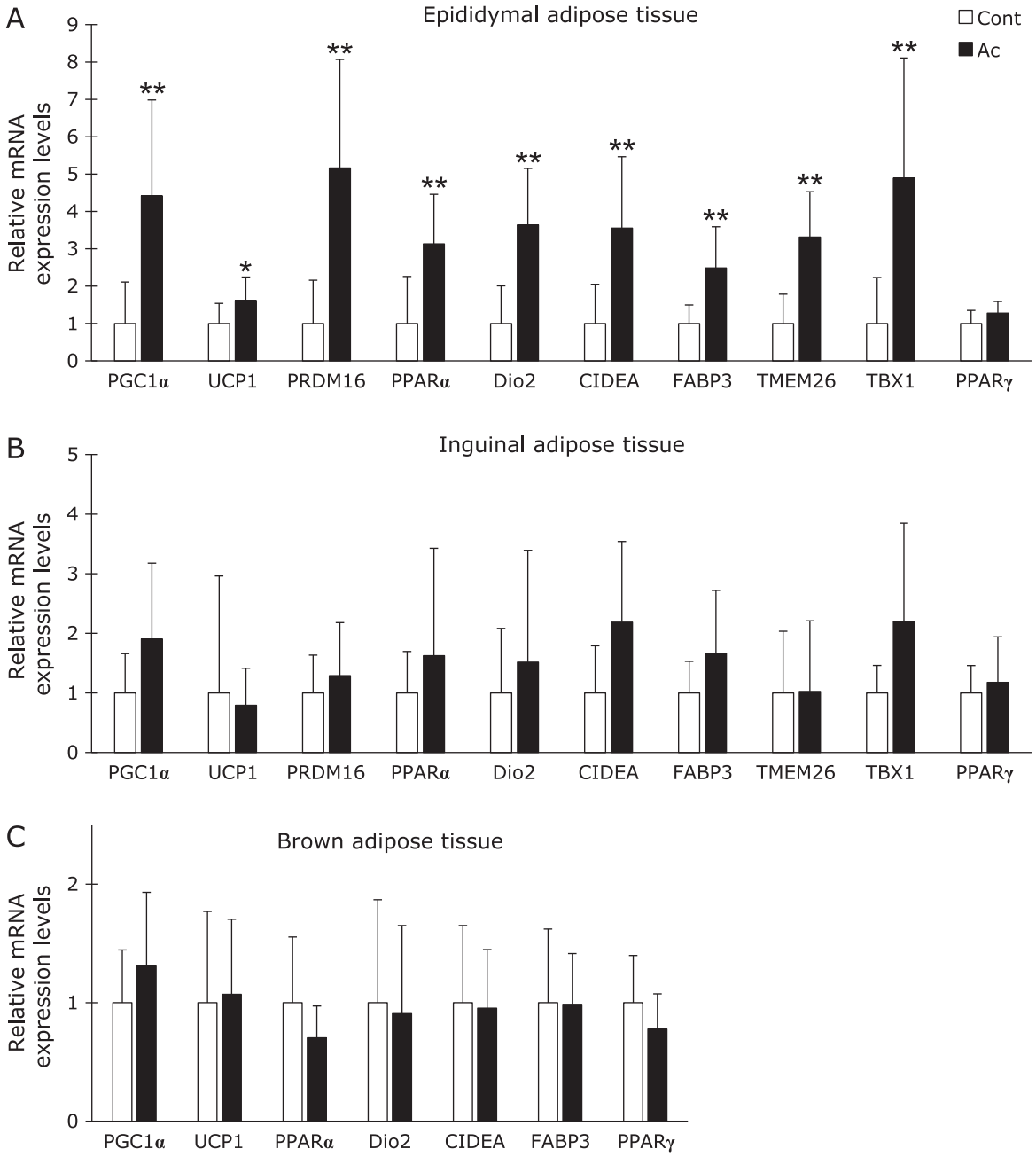

Fig. 3. Effects of acetate on the expression of genes involved in beige adipogenesis in adipose tissue of KK-Ay mice. Relative mRNA expression levels of the indicated genes in (A) epididymal WAT (n = 7–10 per group), (B) inguinal WAT (n = 7–9 per group), and (C) interscapular BAT (n = 7–9 per group) are shown. Differences between the means of two groups were analyzed using Welch's t test. *p<0.05, **p<0.05 vs control group. Cont, control group; Ac, acetate group.

group, although the reason is unclear. To our knowledge, no previous studies have reported the effects of acetate on drinking water intake. Future work should evaluate this using other mouse models.

Beige adipocytes have been reported to be most abundant in subcutaneous WAT.[6] However, several reports have shown that "browning" could be selectively induced in visceral WAT.[32,33] In the present study, we observed that acetate-induced changes in gene expression occurred preferentially in visceral WAT, but not in subcutaneous WAT or BAT. Additionally, significant effects of acetate were observed in 3T3-L1 cells, suggesting that acetate acts directly on adipocytes. Therefore, acetate may induce the expression of genes involved in "browning" by means of specific receptors or adipocytokines that are expressed preferentially in visceral WAT. As one possibility, GPR43 may contribute to the induced expression of genes involved in "browning". GPR43 is a specific receptor for short chain fatty acid, and was previously reported to be highly expressed in WAT but less abundantly expressed in BAT.[20,21] Furthermore, Kimura *et al.*[20] also showed that GPR43 is expressed at higher levels in visceral WAT compared with subcutaneous WAT, and that GPR43 signals induce reduction of visceral WAT by suppressing fat accumulation. Further studies will be required to elucidate the mechanisms of acetate-induced effects on WAT and the contribution of GPR43

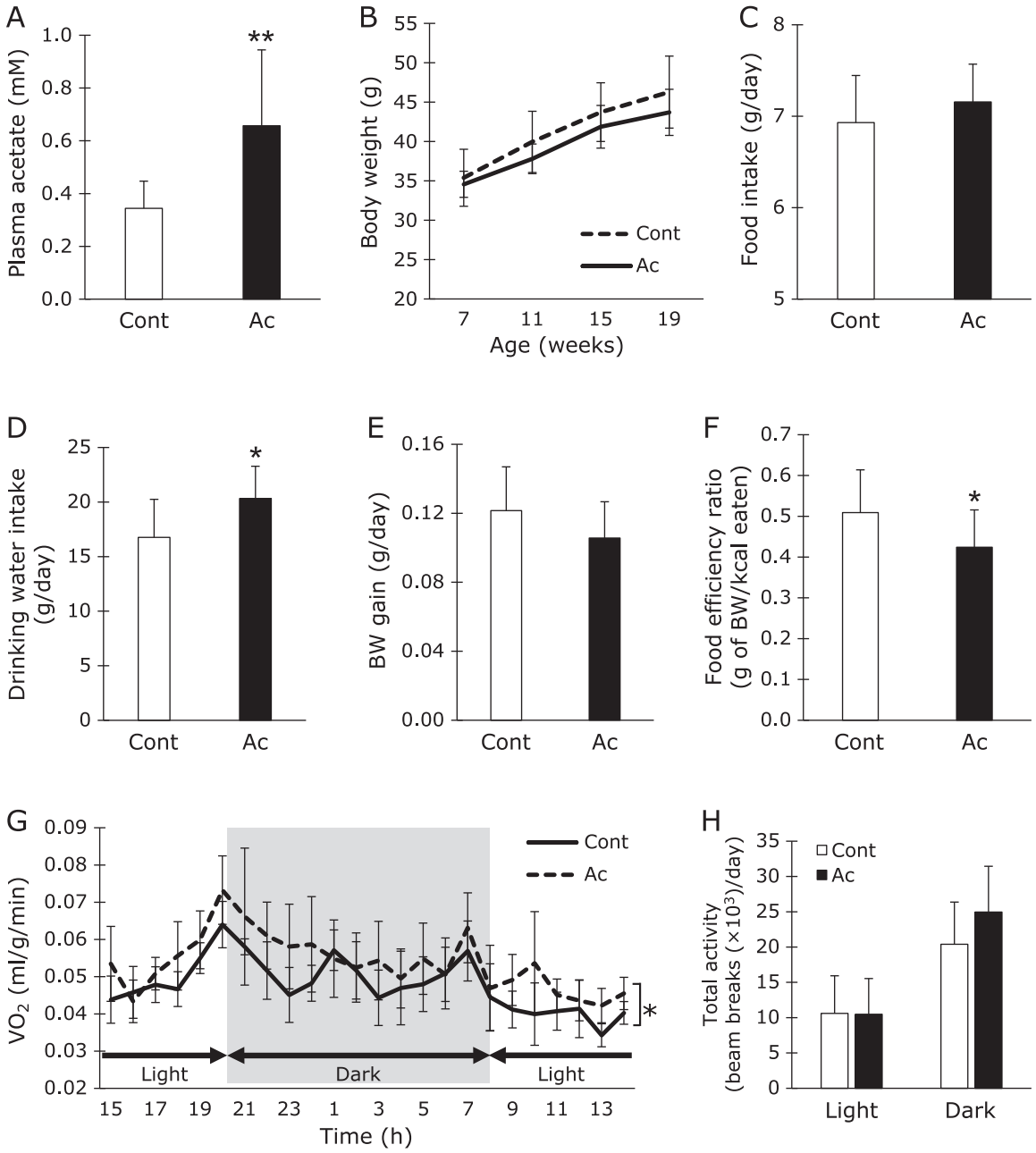

Fig. 4. Effects of acetate administration on the food efficiency ratio and energy expenditure in KK-Ay mice. (A) Plasma acetate concentrations were measured after 8 weeks of treatment (n = 6–10 per group). (B) Body weight (n = 8–12 per group), (C) food intake (n = 12 per group), (D) drinking water intake (n = 12 per group) and (E) BW gain (n = 12 per group) in KK-Ay mice that were treated with vehicle or acetate. (F) The food efficiency ratio (g of BW/kcal eaten) was calculated as described in methods (n = 12 per group). (G) The whole body oxygen consumption rate and (H) the amount of activity were measured after 14 weeks of treatment using a metabolic chamber (n = 5–6 per group). Differences between means were analyzed using Welch's t test (A–F, H) and two-way repeated ANOVA (G). *p<0.05, **p<0.01 vs control group. Cont, control group; Ac, acetate group.

to this process.

Conspicuous increases of multilocular UCP1-positive adipocytes that are common among "browning" are induced by cold exposure, transgenic expression of *PRDM16* and other stimuli.[6,8] A recent report showed that beige adipogenesis could be induced by transdifferentiation from mature white adipocytes or *de novo* differentiation from preadipocytes in interstitial areas. In transdifferetiation, reduction of adipocyte size and the expression of UCP1 protein were induced at early stages of the "browning" process.[6,34–36] In the present study, reduced adipocyte size and the appearance of UCP1-positive adipocytes and interstitial areas with multilocular adipocytes could be observed in visceral WAT of acetate-treated mice. Compared with the conspicuous increase of multilocular adipocytes observed in previous studies,[6,8] histological changes induced by acetate were clearly less. These findings suggest that acetate administration might trigger processes that favored "browning" of visceral WAT. Future studies are needed to fully evaluate the potential benefits of this process.

In conclusion, chronic acetate administration induced the expression of beige adipogenesis-related genes in both 3T3-L1

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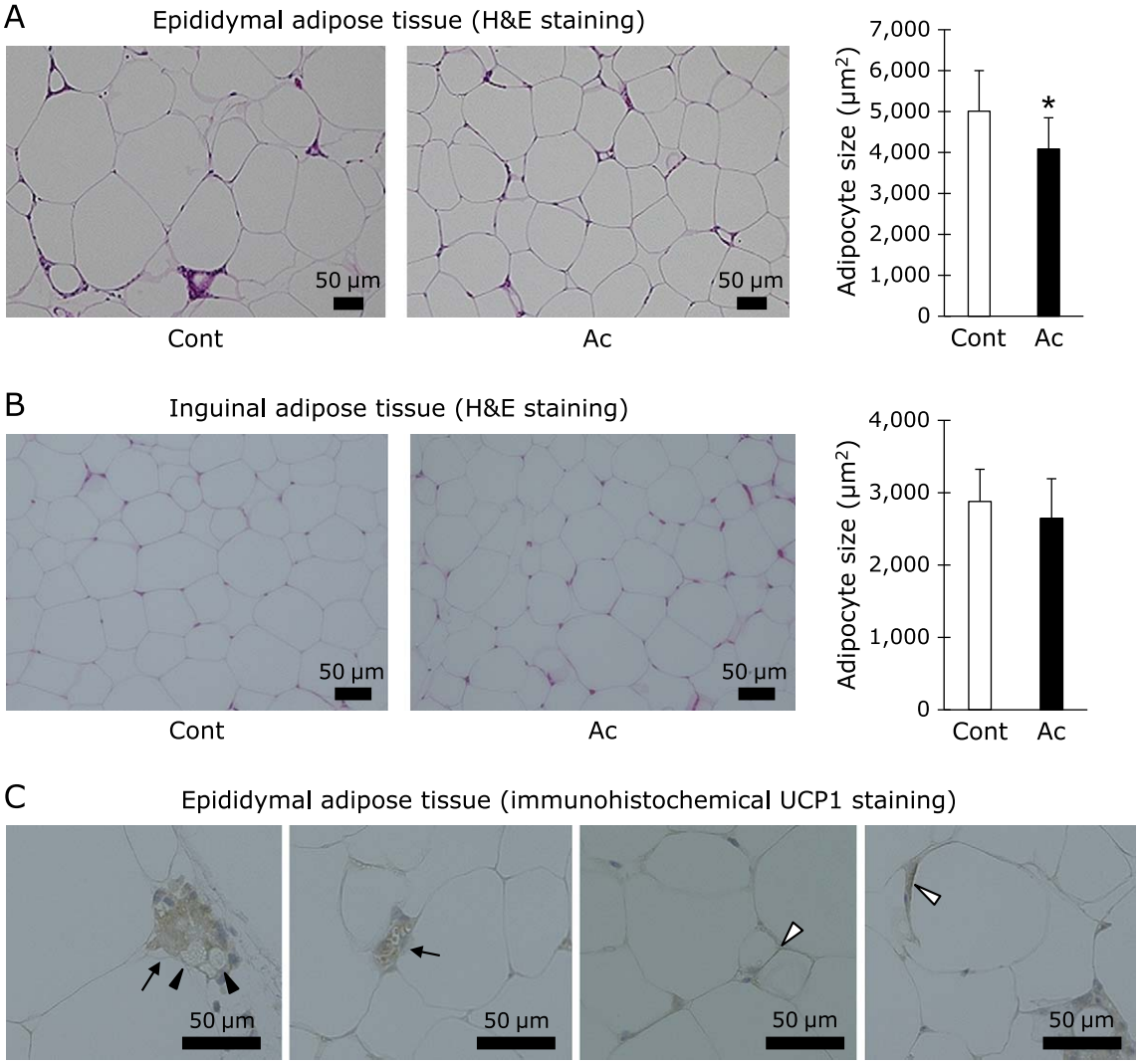

Fig. 5. Effects of acetate administration on adipocyte size and UCP1 expression in epididymal adipose tissue from KK-Ay mice. (A and B) Representative hematoxylin and eosin (H&E) staining of indicated WATs from control and acetate-treated mice. Quantification of adipocyte size in epididymal (A; $n = 9$ per group) and inguinal WAT (B; $n = 3$ per group) are shown in graphs on the right side. (C) Representative images of immunohistochemical UCP1 staining (brown) in epididymal WAT from acetate-treated mice. Black arrows and arrowheads indicate UCP1-positive interstitial areas and multilocular adipocytes, respectively. The white arrowheads indicate UCP1-positive adipocytes. Differences between the means of two groups were analyzed using Welch's *t* test. *$p<0.05$ vs control group. Cont, control group; Ac, acetate group.

cells and visceral WAT of KK-Ay mice. Further studies of the actions of SCFAs, including acetate and its related derivatives, may lead to novel dietary therapies and pharmacological approaches for the treatment of obesity and related disorders.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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