Combination of Exercise and Intake of Amino Acid Mixture Synergistically Induces Beige Adipocyte Formation in Mice

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Summary Exercise combined with dietary factors may have significant effects on the suppression of body fat accumulation. Several trials suggest that amino acid mixtures containing alanine, arginine, and phenylalanine (ARF) combined with exercise can significantly reduce body fat accumulation in overweight adults and high-fat diet-induced obesity in mice. We therefore hypothesized that combining ARF and exercise would significantly induce beige adipocyte formation and that this would contribute to reducing body weight, whereas administration of ARF or exercise alone would not. Administration of ARF (1 g/kg body weight, daily) combined with exercise (5 sessions per week) for 4 wk significantly induced formation of beige adipocytes in inguinal white adipose tissue (iWAT) in mice, although ARF or exercise alone did not. Metabolomic analysis showed that plasma lactate concentration was significantly elevated in the exercise+ARF group relative to the exercise group. Furthermore, lactate dehydrogenase B, which increases redox stress by converting lactate to pyruvate in iWAT and triggers induction of uncoupling protein 1 expression was significantly upregulated in iWAT of the exercise+ARF group. These findings demonstrate the unique effect of ARF combined with exercise for inducing beige adipocyte formation, which may be associated with the suggested lactate-mediated pathway. Appropriate mixtures of amino acids could be used as a dietary supplement before exercise and contributed to increasing energy expenditures.

Key Words alanine, arginine, brown-like adipocyte, phenylalanine, treadmill, metabolome, uncoupling protein 1

Exercise (EX) provides various benefits for overall metabolic health. Regular EX is a particularly effective strategy for body weight control because it elevates energy expenditure (1) and improves overall glucose and lipid homeostasis as well as insulin sensitivity (2, 3). EX combined with dietary factors may have significant or synergistic effects on increasing energy expenditure and suppression of body fat accumulation. For example, in a high-fat diet-induced obesity model in mice, a combination of EX and decaffeinated green tea

extract significantly decreased the accumulation of white adipose tissue (WAT) and improved insulin sensitivity (4). However, treatment with EX or decaffeinated green tea extract alone did not produce significant effects.

Another possible dietary factor that may offer improved health benefits when combined with EX is amino acids. There are many reports that dietary amino acid supplementation can modulate various metabolic changes (5, 6). Recent studies have shown that an amino acid mixture containing alanine, arginine, and phenylalanine (ARF) combined with EX significantly reduced body fat accumulation in overweight adults (7, 8) and diet-induced obesity in mice (9). However, there are few reports that other amino acid mixtures combined with EX reduced body fat accumulation, and the molecular mechanisms of the significant effect of ARF combined with EX on body fat in both obese mice and humans is not clear.

Mammals possess two types of adipose tissue, WAT and brown adipose tissue (BAT), which have physiologically distinct functions. WAT stores excess energy as triglycerides, whereas BAT releases excess energy through heat production from mitochondria (10). Thermogenesis in BAT requires the action of thermogenic uncoupling protein 1 (UCP1), which causes mitochondrial proton leak. Under these circumstances, heat is gener-

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Abbreviations: ARF, amino acid mixture containing alanine, arginine, and phenylalanine; BAT, brown adipose tissue; CE-TOFMS, capillary electrophoresis time-of-flight mass spectrometry; EDL, extensor digitorum longus; eWAT, epididymal white adipose tissue; EX, exercise; FGF21, fibroblast growth factor 21; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin & eosin; iWAT, inguinal white adipose tissue; LDHB, lactate dehydrogenase B; MCT1, monocarboxylic acid transporter 1; MCT4, monocarboxylic acid transporter 4; Nrf2, nuclear factor-erythroid 2-related factor 2; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator 1 α ; scWAT, subcutaneous white adipose tissue; SNS, sympathetic nervous system; TA, tibialis anterior; TBP, TATA box binding protein; UCP1, uncoupling protein 1; WAT, white adipose tissue.

ated instead of ATP (11). Brown-like adipocytes (also called beige adipocytes), which are induced in WAT, release excess energy as heat through a process mediated by UCP1, as in BAT (12). The induction of beige adipocyte can occur in response to β 3-adrenergic receptor agonists, full peroxisome proliferator-activated receptor γ agonists, or simply chronic cold conditions (11, 13, 14). Beige adipocyte induction is also a possible therapeutic target for treating obesity and various related disorders, and the development of beige adipocytes has also been linked by many studies to various dietary factors. In recent years, several published reports have shown that EX induces the formation of beige adipocytes in subcutaneous WAT (scWAT) in rodents (15, 16). In human studies, several reports have also indicated that high intensity-EX induces beige adipocyte formation in scWAT (17, 18). Based on these findings, it is likely that a combination of dietary factors and EX may effectively induce beige adipocyte formation in WAT, thus contributing to body weight control or providing other EX-mediated health benefits. However, little is known about how dietary factors interact with EX to induce beige adipocyte formation.

Accordingly, this study was conducted to examine whether ARF combined with EX significantly induces beige adipocyte formation and whether ARF administration or EX alone do not, as well as to clarify possible mechanisms by which ARF combined with EX mediate beige adipocyte formation.

MATERIALS AND METHODS

Chemicals. ARF was provided by Meiji Co., Ltd. (Tokyo, Japan). The composition of ARF was as follows (weight %): L-alanine, 25%; L-arginine, 25%; L-phenylalanine; 50%. Each amino acid (purity >99%) was obtained from Kyowa Hakko Bio Co., Ltd. (Tokyo, Japan). Anti-UCP1 antibody (ab209483) was acquired from Abcam (Tokyo, Japan). Antibodies of monocarboxylate transporter 1 (MCT1, 20139-1A-AP), monocarboxylate transporter 4 (MCT4, 22787-1-AP), and lactate dehydrogenase B (LDHB, 19988-1-AP) were acquired from Proteintech (Tokyo, Japan). Anti- β -actin antibody (4967) was obtained from Cell Signaling Technology Japan (Tokyo, Japan). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (016-25523) was acquired from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Animal experiments. The design of the animal experiments was approved by the Animal Experiment Committee of Chubu University, and their guidelines were followed in the maintenance of all mice used in this work (Permission No. 3010024).

Effect of ARF combined with EX training on the formation of beige adipocytes in mice. Four-week-old male C57BL/6J mice were acquired from Japan SLC, Inc. (Hamamatsu, Japan), housed in an animal room at $23 \pm 3^\circ\text{C}$ with a 12-h light/dark cycle (illuminated 08:00–20:00), and provided free access to a standard laboratory diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water (19–22). After 1 wk, mice were assigned to

one of four groups ($n=10$, for each group): sedentary control, sedentary control+ARF, EX, and EX+ARF. Mice received oral administration of either vehicle (saline, for control and EX groups) or ARF (1 g/kg body weight, for ARF and EX+ARF groups) daily for 4 wk. The dose of ARF was based on the results of a previous study (9) and a preliminary experiment to show that the administration level did not affect food intake. The EX and EX+ARF group mice were trained for 60 min, five times/wk for 4 wk with a 0% incline treadmill (LE8710MTS; Panlab-Harvard Apparatus, Cornell, Spain, and KN-73; Natsume Seisakusho Co., Ltd., Tokyo, Japan). EX intensity for the five sessions per week was increased as follows: in the first week, 15 m/min; in the second week, 17.5 m/min; and in the final 2 week, 20 m/min. We performed a preliminary experiment to confirm that the EX intensity and duration did not induce beige adipocyte formation in inguinal WAT (iWAT) and UCP1 expression in BAT. During the experimental period, all mice groups were allowed free access to water and AIN-93G diet (23). At 16 h after the last EX training session and administration of saline or ARF, blood samples were collected from isoflurane-anesthetized mice, using a syringe containing heparin; plasma was subsequently isolated by centrifugation. Then, the iWAT, epididymal WAT (eWAT), interscapular brown adipose tissue (BAT), gastrocnemius+soleus muscle, and extensor digitorum longus (EDL)+tibialis anterior (TA) muscles were also removed (19–22). Following the protocol from our previous reports (19–22), small samples of adipose tissues were fixed and used for both UCP1 immunostaining and hematoxylin & eosin (H&E) staining. Aliquots of the tissues were also homogenized and used for immunoblotting analysis of UCP1 protein in iWAT and BAT, with GAPDH or β -actin used as controls, following the methods employed in our previous reports (19–22).

Metabolomic analysis. Metabolomic analyses were conducted on plasma from the EX and EX+ARF groups using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis with an Agilent CE-TOFMS system (Agilent Technologies, Tokyo, Japan) by Human Metabolome Technologies Inc. (Tsuruoka, Japan). Briefly, 50- μL plasma samples were extracted with 200 μL of methanol and 20 μM of internal standard (camphor-10-sulfonic acid, for anion analysis, and methionine sulfone, for cation analysis), to which 150 μL of Milli-Q water was added before samples were mixed. The samples were filtered with a 5-kDa cut-off filter at $9,100 \times g$ and 4°C for 120 min. After filtrates were dried, they were dissolved in 50 μL of water and subjected to CE-TOFMS analysis (24). Automatic integration software (MasterHands ver. 2.17.4.19, Keio University, Tsuruoka, Japan) was used to process the detected peaks, which were aligned based on their m/z values, peak areas, and normalized migration times. Putative metabolites from the Human Metabolome Technologies metabolite database were used to annotate the peaks, internal standards were used to normalize peak areas, and comparisons of the peak areas against

Fig. 1. Representative H&E and immunohistochemical staining images of (A) iWAT and (B) BAT from mice in the control, ARF, EX, and EX+ARF groups for 4 wk.

calibration curves generated using internal standardization techniques were used to quantify the metabolites, thus enabling a comparison of the metabolites between the EX and EX+ARF groups.

Measurement of plasma fibroblast growth factor 21 (FGF21) concentration. Plasma FGF21 concentration was measured using an ELISA kit (Mouse/Rat FGF21 Quantikine ELISA Kit, R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Measurement of mRNA levels. Total RNA isolation from the iWAT samples and mRNA transcript level assays with an ABI PRISM7300 real-time PCR system (Thermo Fisher Scientific, Yokohama, Japan) were performed following methods used by previous studies (19, 25). TaqMan Gene Expression Assays with the following ID numbers were used: MCT1 (gene name, *Slc16a1*), Mm01306379_m1; MCT4 (gene name, *Slc16a3*), Mm00446102_m1; LDHB, Mm00493146_m1; TATA box binding protein (TBP), Mm00446971_m1; FGF21, Mm00840165_g1.

Immunoblot analysis of MCT1, MCT4, and LDHB proteins. Immunoblot sample preparation from iWAT, gastrocnemius+soleus muscle, and EDL+TA muscle samples and immunoblot analysis was performed following methods used by previous studies (19–22, 25). Immunoreactivity for LDHB was visualized using Pierce Western Blotting Substrate (Thermo Fisher Scientific), and MCT1 and MCT4 immunoreactivity was visualized using ImmunoStar LD (FUJIFILM Wako Pure Chemical

Corporation) (19–22, 25).

Statistical analyses. All data are expressed as mean $\pm$ SE values. The data presented in Fig. 2 and Table S1 (Supplemental Online Material) were analyzed using two-way ANOVA, followed by the Tukey–Kramer test with a $p < 0.05$ significance threshold. Differences between two group means were assessed using Student's *t*-tests with a $p < 0.05$ significance threshold (Table S2 (Supplemental Online Material), Figs. 3–5 and S2 (Supplemental Online Material)).

RESULTS

ARF combined with EX significantly induced beige adipocyte formation in iWAT

We first examined whether administration of ARF combined with EX for 4 wk synergistically induced beige adipocyte formation compared with ARF or EX alone in mice. There were no significant interaction effects between the two treatments (ARF and EX) or differences among the four groups in body weight gain, food intake, adipose tissue (i.e., iWAT, eWAT, or BAT) or skeletal muscles weights (Table S1). Based on H&E-stained iWAT samples, the ARF alone and EX alone groups did not show clear visible multilocular adipocytes, indicating a lack of beige adipocytes, and UCP1 immunostaining was also negative (Fig. 1A). In contrast, distinct multilocular adipocytes were visible in H&E stains of iWAT from the EX+ARF group. In addition, UCP1-immunopositive cells were also distinctly

Fig. 2. UCP1 protein levels of (A) iWAT and (B) BAT from mice in the control, ARF, EX, and EX+ARF groups for 4 wk. The protein levels are expressed as fold-increases relative to the control group (=1) after normalization with (A) GAPDH or (B) β -Actin expression levels. Data are presented as mean $\pm$ SE values ($n=9$ or 10). The ARF $\times$ EX interaction effect in (A) was significant ($p<0.05$); values in (A) that do not share a common letter differed significantly ($p<0.05$). The ARF $\times$ EX interaction effect in (B) was not significant (NS).

Fig. 3. The concentration of metabolites associated with glycolysis and the TCA cycle pathway in the plasma of mice in the EX and EX+ARF groups for 4 wk. Data are presented as mean $\pm$ SE values ($n=3$). *Significantly different compared with the EX group ($p<0.05$); ND, not detected.

observed in iWAT from the EX and ARF group compared with the other groups (Fig. 1A). In eWAT from the four groups, there were no multilocular adipocytes, and UCP1 immunostaining was also negative (Fig. S1, Supplemental Online Material). In addition, the four groups did not differ in terms of H&E staining or UCP1 immunostaining of BAT (Fig. 1B).

To confirm the significant immunohistochemical

analysis results observed in the EX+ARF group, we examined the UCP1 protein levels in iWAT. Either administration of ARF alone or EX alone did not significantly induce UCP1 protein expression in iWAT (Fig. 2A). In contrast, the administration of ARF combined with EX significantly induced UCP1 protein expression in iWAT compared with the ARF and EX group (Fig. 2A). However, the UCP1 protein levels of BAT did not

Fig. 4. The mRNA transcript (A) and protein levels (B) of MCT1, MCT4, and LDHB in iWAT of mice in the EX and EX+ARF groups for 4 wk. (A) MCT1, MCT4, and LDHB mRNA transcript levels are expressed as fold-increases relative to the EX group after normalization using TBP mRNA transcript levels. (B) MCT1, MCT4, and LDHB protein levels are expressed as fold-increases relative to the EX group (=1) after normalization with GAPDH protein expression level. Data are presented as mean $\pm$ SE values ($n=9$). * Significantly different compared with the EX group ($p<0.05$).

Fig. 5. The plasma concentrations (A) and mRNA transcript (B) of FGF21 in iWAT of mice in the EX and EX+ARF groups for 4 wk. (B) mRNA transcript levels are expressed as fold-increases relative to the EX group after normalization using TBP mRNA transcript levels. Data are presented as mean $\pm$ SE values ($n=9$).

differ among the four groups (Fig. 2B).

Plasma lactate and TCA cycle intermediates significantly increased under ARF combined with EX

The significant induction of beige adipocyte formation in iWAT of the EX+ARF group raised the issue of how ARF combines with EX training to induce beige adipocyte formation. To elucidate this mechanism, metabolic changes were assayed by CE-TOFMS in order to identify significantly differing levels of metabolites. Thus, we analyzed the metabolite profiles of plasma from the EX and EX+ARF groups and compared them. An overview of the plasma metabolite profiles revealed 186 peaks (118 cations and 68 anions) using the anion and cation modes of CE-TOFMS. Among the detected peaks, concentrations (μ M) of 58 peaks (42 cations and 16 anions) were calculated (Table S2). The metabolomic analysis clearly indicated significant changes had occurred in central carbon metabolism (i.e., glucose metabolism). As shown in Fig. 3, the concentrations of

2-oxoglutarate (2.2-fold), malate (2.1-fold), lactate (1.7-fold), and fumarate (1.6-fold) were significantly increased in the EX+ARF group compared with the EX group. Pyruvate was increased (1.3-fold) in the EX+ARF group; however, the increase was not significant. In addition, the concentrations of Ala (1.9-fold), Ser (1.8-fold), and Gly (1.3-fold), which are converted into pyruvate and can further be metabolized into lactate, were significantly increased in the EX+ARF group. *ARF combined with EX significantly induced LDHB expression in iWAT*

The metabolomic analysis indicated that plasma lactate was significantly increased in the EX+ARF group. Previous studies have demonstrated that lactate induces beige adipocytes mediated by a change in the intracellular redox state (26–28). Incorporated lactate via MCTs and/or intracellular lactate are metabolized into pyruvate by LDHB. The LDHB-mediated transformation induces a higher $\text{NADH:H}^+/\text{NAD}^+$ ratio and triggers

upregulation of UCP1 expression to alleviate redox stress (26–28).

Accordingly, we examined the effect of ARF combined with EX on the expression of MCT1, MCT4, and LDHB in iWAT and muscles. The mRNA and protein expression levels of LDHB were significantly increased in the EX+ARF group compared with the EX group (Fig. 4A and B). The mRNA transcript and protein expression levels of MCT1 and MCT4 were not different between the groups (Fig. 4A and B). In addition, the MCT1 and MCT4 protein expression levels were not different between the groups in either the gastrocnemius+soleus muscle or EDL+TA muscle (Fig. S2A and B).

ARF combined with EX did not affect FGF21 plasma concentration or transcript abundance in iWAT

Previous studies have demonstrated that FGF21 induces the formation of beige adipocytes (29) and that EX significantly increases serum FGF21 concentration (30, 31). FGF21 is also expressed in WAT, and it is possible to induce beige adipocyte formation via autocrine or paracrine system (32). Therefore, we examined the effect of ARF combined with EX on the plasma FGF21 concentration and mRNA level in iWAT. The plasma FGF21 protein concentration and mRNA abundance in iWAT did not differ between the groups (Fig. 5A and B).

DISCUSSION

EX is considered an effective strategy for realizing various health benefits. However, it may be difficult for humans that have various forms of heart disease or physical disability to enhance their EX intensity. Accordingly, EX combined with dietary factors may be expected to increase energy expenditure and enhance other EX-related biological activity. Recent studies have demonstrated that ARF combined with EX can significantly reduce body fat accumulation in overweight adults (7, 8) and diet-induced obesity in mice (9). We hypothesized that a combination of ARF and EX would significantly induce beige adipocyte formation and may contribute to control of body weight.

After confirming that the EX intensity and duration did not affect beige adipocyte formation in iWAT, we examined whether a combination of ARF and EX significantly induce beige adipocyte formation. This study demonstrated that ARF administration combined with EX for 4 wk could significantly induce beige adipocyte formation in iWAT in mice, even though neither ARF administration nor EX alone had such an effect. The mice used in this study did not receive a high-fat diet nor do they represent a genetically induced obesity model. Therefore, it is not unexpected that body weight gain and WAT deposits did not differ among the groups. It is also reasonable that beige adipocyte formation is induced in iWAT but not in eWAT. PRD1-BF-1-RIZ1 homologous domain-containing protein-16, which is essential for the induction of beige adipocytes (33, 34), is highly expressed in scWAT (i.e., iWAT), but is expressed at an extremely low level in abdominal WAT (i.e., eWAT) (34). In addition, many studies have demonstrated that induction of beige adipocyte forma-

tion in scWAT, not abdominal WAT, is significantly associated with a reduction of body fat accumulation and can be a potential therapeutic intervention against obesity (35–38). These previous findings and the current results suggest that the reduction of body fat accumulation promoted by ARF combined with EX in overweight adults (7, 8) and diet-induced obesity in mice (9) may be associated with the induction of beige adipocyte formation.

These results raise the issue of how ARF treatment combines with EX to induce the formation of beige adipocytes in iWAT of mice. One of the possible mechanisms, the β 3-adrenergic signaling pathway via the sympathetic nervous system (SNS), can induce UCP1 expression in iWAT and BAT (39). EX can stimulate SNS (40) and increase plasma norepinephrine concentrations (41). Furthermore, BAT is known to be more sensitive and dominant than WAT with respect to activation of the β 3-adrenergic signaling pathway via the SNS (42–44). If the β 3-adrenergic signaling pathway via the SNS is activated by ARF combined with EX, a significant induction of UCP1 expression should be observed in BAT from the ARF+EX group. However, in the present study, the UCP1 protein levels of BAT did not differ among the four groups. Therefore, it is unlikely that induction of beige adipocyte formation by ARF combined with EX is associated with activation of the β 3-adrenergic signaling pathway via the SNS.

Another possible factor underlying the mechanism of beige adipocyte induction by ARF combined with EX is FGF21. Previous studies have demonstrated that FGF21 induces the formation of beige adipocytes (29) and that EX significantly induces serum FGF21 concentration through upregulation of mRNA transcript abundance in the liver (30, 31). In addition, FGF21 is also expressed in WAT; however, FGF21 expressed in WAT acts locally via the autocrine or paracrine system rather than contributing to plasma concentrations of FGF21 (45–47). In the present study, plasma FGF21 concentrations did not differ between the EX and EX+ARF groups, suggesting that the EX+ARF treatment does not affect FGF21 expression in the liver. In addition, FGF21 transcript abundances in iWAT did not differ between the EX and EX+ARF groups. Although FGF21 may induce beige adipocyte formation in WAT via the autocrine or paracrine system (32), the present results suggest that induction of beige adipocyte formation ARF combined with EX is not associated with FGF21.

Metabolomic analysis is a particularly useful tool for exploring alternative factors of beige adipocyte induction by ARF combined with EX, and it can provide overviews of metabolic changes that may elucidate underlying physiological mechanisms. In the present study, no significant changes were observed in UCP1 expression and immunostaining between the ARF group compared with the control and EX groups. To determine how EX+ARF induced beige adipocyte formation, we focused on the significant changes in plasma metabolites and several parameters in iWAT between the EX and EX+ARF groups. Although the metabolome results could be used

to compare the two groups, focusing on the comparison between the two groups is sufficient to explain the possible mechanisms by which EX combined with ARF exerted a significant effect. Our metabolome results notably showed that the plasma concentration of lactate was significantly elevated in the EX+ARF group compared with the EX group, despite this analysis having been conducted more than 16 h after the final EX session and ARF administration. Although we did not perform metabolomic analysis to compare the control and ARF groups, ARF alone did not significantly affect the plasma lactate concentration (control, 2.61 ± 0.20 mM; ARF, 2.54 ± 0.21 mM). These results suggest that the plasma lactate concentration was significantly increased by the ARF combined with EX, not ARF alone. Lactate has been long considered a waste product of anaerobic metabolism. However, lactate production is now regarded to enhance glucose metabolism under aerobic conditions (48, 49), suggesting that ARF combined with EX accelerates glucose metabolism. Carrière et al. demonstrated that lactate induces beige adipocyte formation mediated through a change in the intracellular redox state (26–28). In adipocytes, lactate is metabolized into pyruvate by LDHB (lactate + $\text{NAD}^+ \rightarrow$ pyruvate + $\text{NADH} + \text{H}^+$); this LDHB-mediated transformation induces a higher $\text{NADH:H}^+/\text{NAD}^+$ ratio (i.e., a high redox state) (26–28). This increase triggers induction of UCP1 expression via redox-sensitive signaling pathways (50–52). The $\text{NADH:H}^+/\text{NAD}^+$ ratio triggered elevation of mitochondrial redox state may be associated with nuclear factor-erythroid 2-related factor 2 (Nrf2), a transcription factor controlling a broad range of reactive oxygen species and thiol targeted antioxidant enzymes (52, 53). Significant induction of UCP1 expression was observed in iWAT, but administration of *N*-acetylcysteine suppressed the induction in Nrf2 knock out mice (53). As a result, uncoupling activity mediated by UCP1 promotes electron transport chain activity, resulting in a decrease in the $\text{NADH:H}^+/\text{NAD}^+$ ratio, which alleviates redox stress (26–28).

In this study, the plasma lactate concentration was significantly elevated in the EX+ARF group compared with the EX group. Furthermore, the LDHB protein expression level was significantly increased in iWAT from the EX+ARF group compared with that from the EX group. This significant increase may be associated with the beige adipocyte formation induced by the combination of ARF and EX, that is, lactate can be converted into pyruvate by LDHB and facilitates elevation of mitochondrial redox state through increase in $\text{NADH:H}^+/\text{NAD}^+$ ratio, thus UCP1 is induced in iWAT from the EX+ARF group. Nrf2 can be associated with the induction in the EX+ARF group (53). Peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) is one of the key factors of metabolic controls. Summmatter et al. reported that PGC-1 α promotes LDHB transcription by coactivating estrogen-related receptor- α on LDHB promoter (54). ARF administration combined with EX may modulate PGC-1 α activity and result in upregulation of LDHB in iWAT. In addition, concen-

trations of Ala, Gly, and Ser, which can be converted into pyruvate and form lactate, were significantly increased in the EX+ARF group compared with the EX group. These significant increases may have contributed to the significant elevation of the plasma lactate concentration observed in the EX+ARF group.

Our metabolome results also showed that the plasma concentrations of Arg, Phe, 2-oxoglutarate, and fumarate were significantly elevated in the EX+ARF group compared with the EX group. Increases in Arg and Phe content could be converted into 2-oxoglutarate or fumarate, and this conversion may be associated with significant increases in plasma 2-oxoglutarate and fumarate concentrations in the EX+ARF group.

The results of this study raise some unaddressed questions and key limitations. One report showed that 2-oxoglutarate induced beige adipocyte formation in WAT in mice (55). Although 2-oxoglutarate may be another possible factor affecting beige adipocyte induction by ARF combined with EX, we did not investigate whether the increased plasma 2-oxoglutarate content mediated beige adipocyte formation in the EX+ARF group. In addition, because only small amounts of iWAT were obtained from the mice, we were unable to determine $\text{NADH:H}^+/\text{NAD}^+$ ratio and lactate content in iWAT. In future research, using iWAT-specific LDHB knockout mice or inducing lactate depletion with chemical inhibitors may be useful tools for confirming lactate-mediated beige adipocyte formation is induced by ARF combined with EX.

In conclusion, we have demonstrated that ARF administration combined with EX for 4 wk can significantly induce beige adipocyte formation in iWAT of mice, even though ARF or EX alone did not have this effect. A lactate-mediated pathway of beige adipocyte formation may be driven by administration of ARF combined with EX. These findings demonstrate that the combination of ARF with EX offers a unique biological response for inducing beige adipocyte formation, which may be involved with increasing energy expenditures.

Authorship

TT and TK designed this research; TK and NE performed the experiments; TT wrote the paper.

Disclosure of state of COI

No conflicts of interest to be declared.

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Supporting information

Supplemental online material is available on J-STAGE.

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