Exercise-inducible factors to activate lipolysis in adipocytes

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EXCESSIVE LIPID ACCUMULATION in adipocytes is a central feature of obesity and metabolic syndrome. Excess energy is primarily stored as triacylglycerol (TAG) in the lipid droplets (LDs) of mammalian adipose tissue, and those TAG reserves are hydrolyzed to supply fatty acids (FAs) to various tissues by a process called lipolysis, when the stored energy is required such as during starvation and exercise. Thus, physiological strategies such as exercise training aimed toward fat loss by active lipolysis in adipocytes (i.e., fat mobilization) and FA oxidation in muscles (i.e., fat utilization) have become preferred therapeutic agents against LD-associated metabolic diseases.

Exercise-inducible factors to activate lipolysis in adipocytes. J Appl Physiol 115: 260–267, 2013. First published May 16, 2013; doi:10.1152/japplphysiol.00427.2013.—We examined the effects of exercise training on the levels of lipid droplet (LD)-associated and mitochondria-related proteins in diet-induced obese (DIO) rats. Furthermore, we assessed putative factors induced by exercise to activate lipolysis in differentiated 3T3-L1 adipocytes. DIO Wistar male rats (age 20 wk) were divided into sedentary control (SED, n = 7) and exercise training (EX, n = 7) groups. EX animals were subjected to treadmill running (25 m/min, 1 h/day, 5 days/wk) for 6 wk. Epididymal fat was dissected and used for protein analyses. 3T3-L1 adipocytes were incubated with media containing hydrogen peroxide (H2O2), sodium-lactate, caffeine, AICAR, or SNAP (NO donor) for 6 h, or 1 mM H2O2 for 15 min, followed by incubation with normal media for up to 24 h total. Protein expression levels and lipolytic activities were biochemically assayed. Epididymal fat significantly decreased in EX animals compared with SED animals. Levels of cytochrome c oxidase (COx), perilipin, hormone sensitive lipase (HSL), and adipose triglyceride lipase (ATGL) proteins in epididymal fat pads of EX animals were significantly increased compared with those in SED animals. In 3T3-L1 cells, glycerol or fatty acid release was significantly increased by all treatments. Lactate or SNAP significantly increased PGC-1α expression, and H2O2 significantly increased COX protein levels compared with controls. Expression of perilipin, HSL, ATGL, or comparative gene identification (CGI)-58 was significantly increased by all treatments. By increasing lipolytic activity in adipocytes, the exercise-inducible factors are attractive therapeutic effectors against LD-associated metabolic diseases.
muscle cells is dependent on ROS production (34). Similarly, it was demonstrated in a mouse fibroblastic cell line that \( \text{H}_2\text{O}_2 \) treatment increased PGC-1\( \alpha \) through increased cAMP response element-binding protein (CREB), the important transcription factor for PGC-1\( \alpha \) gene expression (3, 35). Furthermore, in mouse skeletal muscle, Koves et al. demonstrated that either acute exercise or PGC-1\( \alpha \) overexpression stimulated gene expression of HSL, ATGL, CGI-58, and perilipin 5. Perilipin 5 belongs to a family of lipid coat protein enriched in oxidative muscles, suggesting that there is an intimate molecular connection between LD regulation and mitochondrial metabolism (20). As a whole, these findings led us to suspect that these exercise-inducible factors, known to be generated in skeletal muscle, could travel from muscle to adipose tissue and/or could be generated in adipose tissue, thereby increasing lipolytic activity in adipocytes by means of increased mitochondrial biogenesis and LD-associated proteins. This idea was tested in the investigation reported here.

Here we examined the effects of exercise training on levels of LD-associated proteins and mitochondrial biogenic signaling in adipose tissue of exercised diet-induced obese (DIO) rats, in which enhanced lipolytic activity would be more appreciable than in nonobese rats. We also examined the effects of compounds that are known to reproduce some of the exercise effects in skeletal muscle such as \( \text{H}_2\text{O}_2 \), sodium-lactate, caffeine, AICAR, and the NO donor acetyl-penicillamine (SNAP), on the expression of LD-associated proteins and mitochondrial biogenic signaling to explore putative factors induced by exercise training to activate lipolysis in differentiated 3T3-L1 adipocytes. We hypothesized that exercise training would increase the levels of LD-associated proteins and mitochondrial biogenic signaling in DIO rats. Furthermore, we hypothesized that ROS, lactate-inducible signals, increased intracellular \( \text{Ca}^{2+} \) levels, AMPK, and NO would increase lipolytic activity in adipocytes.

MATERIALS AND METHODS

Animal care. Ethical approval for this study was obtained from the Committee on Animal Care at the University of Tsukuba and Ritsumeikan University. Male Wistar rats (220–250 g, 10 wk old) were obtained (Charles River Japan, Kanagawa, Japan) and cared for according to the guiding principles for the care and use of animals based on the Declaration of Helsinki. The rats were housed individually in an animal facility under controlled conditions (12:12-h light-dark cycle). To induce obesity and hyperglycemia, the rats were fed the high-sucrose diet during the 6-wk experimental period. Exercise intensity was kept constant during the training period. Rodent treadmill at a speed of 30 m/min for 30 min at 30 m/min without incline. Immediately after exercise, epididymal fat was quickly removed, weighed, rinsed in ice-cold saline, and frozen in liquid nitrogen.

Acute exercise protocol. Male Wistar rats (220–250 g, 10 wk old) were divided into sedentary control (CON, \( n = 6 \)) and acute exercise (AEX, \( n = 6 \)) groups. Prior to the acute exercise, rats were accustomed to the treadmill at a speed of 30 m/min for 30 min. One week after the acclimation, the rats of the AEX group ran on the treadmill for 30 min at 30 m/min without incline. Immediately after exercise, epididymal fat was quickly removed, weighed, rinsed in ice-cold saline, and frozen in liquid nitrogen.

Cell culture protocol. All reagents for cell culture were obtained from Wako (Osaka, Japan) unless otherwise mentioned. The cell culture procedure was the same as that described in our previous report (17). Briefly, 3T3-L1 cells were maintained in DMEM/10% fetal bovine serum (FBS). For differentiation, confluent cells (day 0) were treated with differentiation media, which is a hormone mixture containing 1 \( \mu \text{M} \) dexamethasone, 0.5 \( \mu \text{M} \) 3-isobutyl-1-methylxanthine (IBMX), and 5 \( \mu \text{g} /\text{ml} \) insulin in DMEM/10% FBS. After 48 h (day 2), the differentiation media was removed and cells were further cultured in growth media (DMEM/10% FBS supplemented with 5 \( \mu \text{g} /\text{ml} \) insulin). The growth media was replaced with fresh growth media every 3 days up to the experimental days.

Differentiated 3T3-L1 adipocytes were incubated with media containing 10 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), 10 mM sodium-lactate, 5 mM caffeine, 2 mM AICAR (Sigma), 100 \( \mu \text{M} \) SNAP for 6 h, or 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 15 min, followed by incubation with normal fresh media for 18 h (23 h and 45 min for 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \)). After 24 h from the beginning of the incubation with \( \text{H}_2\text{O}_2 \), sodium-lactate, caffeine, AICAR, or SNAP cells were used for assays of lipolytic activity or Western blotting.

Lipolytic stimulation. When lipolytic activity was analyzed, 2% FA-free BSA (Wako) was used instead of 10% FBS. Lipolytic activity was added to differentiated 3T3-L1 adipocytes as follows: after washing twice with Hank’s buffer, cells were incubated with DMEM containing 10% FBS/4mML-glutamine (Gibco)/25 mM HEPES (pH 7.4)/0.5 mM IBMX/10 \( \mu \text{M} \) isoproterenol at 37°C.

Western blotting. Tissue samples (300 mg each) were homogenized in 2 ml of buffer containing 20 mM Tricine (pH 7.8), 250 mM sucrose, 10 mM NaF, 1 mM PMSF, a protease inhibitor mixture (Sigma-Albardh, St. Louis, MO), and phosphatase inhibitor cocktail (Sigma-Albardh), and incubated on ice for 20 min. Tissues were then homogenized using a Potter-Elvehjem tissue homogenizer (AS ONE, Osaka, Japan) on ice with 10 gentle strokes with the motor-driven pestle at 2,500 rpm. Homogenates were mixed with four times the sample volume of cold acetone (−20°C) and incubated overnight at −20°C. The samples were then centrifuged at 9,000 g for 15 min at 4°C, and the supernatants were disposed. Acetone was evaporated and the pellets were resuspended with buffer containing 20 mM Tris (pH 7.4), 1 mM EDTA, and 0.1% Triton X-100, and protein concentration was measured. The protein extracts were subjected to SDS-PAGE and transferred to a nitrocellulose membrane.

3T3-L1 cells were washed with PBS and directly dissolved in the heated SDS-PAGE sample buffer. Aliquots of the extracts were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Proteins were probed with an antibody to CGI-58 (sc-102285; Santa Cruz Biotechnology), HSL (#4107; Cell Signaling), ATGL (#2138; Cell Signaling), perilipin (GPE, Progen, Dara, Australia), PGC-1\( \alpha \) (ST-1202; Calbiochem), electron transport chain protein cytochrome c oxidase (COX) (ab54575; abcam), endothelial NOS (eNOS) (#610297; Transduction Laboratories), phospho-NOS (p-NOS) (sc-12972; Santa Cruz Biotechnology), AMPK (#2933; Cell Signaling), phospho-AMPK (p-AMPK) (#2531; Cell Signaling), calcium/calmodulin-dependent protein kinase II (CaMKII) (#3832; Cell Signaling), phospho-CaMKII (p-CaMKII) (#3831; Cell Signaling) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (G9545; Sigma-Aldrich) detected by the enhanced chemiluminescence method (Amersham Biosciences). The immunoblotted membranes were scanned with an ImageQuant LAS-4000 (GE Healthcare) luminescent image analyzer and the optical density of each specific band was analyzed with ImageQuant ageQuant LAS-4000 (GE Healthcare) luminescent image analyzer and the optical density of each specific band was analyzed with ImageQuant TL software (GE Healthcare). Densitometric analysis of immunoblots was normalized to GAPDH.

Glycerol release, FA release, and triglyceride measurements. 3T3-L1 cells were grown in 12-well dishes. Differentiated cells were pretreated with either 10 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), 10 mM sodium-lactate, 5 mM...
cafeine, 2 mM AICAR, 100 μM SNAP for 6 h, or 1 mM H2O2 for 15 min, and then washed twice with Hank’s buffer and incubated with DMEM containing 2% FA-free BSA (Sigma)/20 mM HEPES (pH 7.4) with or without 0.5 mM IBMX and 10 μM isoproterenol at 37°C. After incubation for 3 h, the media was collected and assayed for glycerol and FA content using the triglyceride E-test kit and NEFA C-test kit, respectively (Wako).

Statistical analysis. Statistical analyses were performed by unpaired t-tests or one-way ANOVA, as appropriate. Bonferroni post hoc test was used as needed, and significance was set at \( P < 0.05 \). All results are presented as means ± SEM.

RESULTS

Effect of exercise training on body weight, epididymal fat weight, mitochondrial biogenic signaling, and LD-associated protein levels. As expected, exercise reduced body weights and epididymal fat pad weights, thereby confirming a physiological effect of exercise (Table 1).

Given that inhibition of mitochondrial respiration by antimycin A and 2,4-dinitrophenol decreased lipolysis in adipocytes (12), we hypothesized that mitochondrial oxidative activity is crucial for active lipolysis in adipose tissue, thereby providing a potential mechanism for an exercise training-mediated decrease in fat pad weight. Thus, we determined levels of mitochondrial biogenic signaling in epididymal fat for SED and EX rats. PGC-1α, a master regulator of mitochondrial biogenesis, did not increase (\( P = 0.1 \)), but COx significantly increased in response to endurance training (Fig. 1).

We also examined expression of LD-associated proteins in epididymal fat in response to exercise training. The EX group showed significantly higher expression of HSL, perilipin, and ATGL in epididymal fat than in the SED group (\( P < 0.05 \)). Expression of CGI-58 in the EX group tended to increase compared with the SED group (\( P = 0.05 \)) (Fig. 2).

Effects of acute exercise on calcium signals, NO, and AMPK responses in epididymal fat. We explored exercise-inducible factors in adipose tissue that may potentially elicit augmented mitochondrial biogenic signaling and LD-associated protein expression by exercise training. Therefore, we examined whether exercise-inducible factors known to be generated in skeletal muscle are also inducible in adipose tissue in response to a single bout of exercise. For this purpose, we assessed the p-NOS/eNOS ratio, p-AMPK/total AMPK ratio, and p-CaMKII/total CaMKII ratio, which would reflect NO production, AMPK activity, and intracellular Ca2+ concentration, respectively, in adipose tissue dissected immediately after exercise. As shown in Fig. 3, acute exercise significantly increased p-NOS/eNOS and p-AMPK/total AMPK ratios, suggesting that NO production and AMPK activity may be increased by a single bout of exercise in adipose tissue.

Effects of putative exercise-inducible factors on mitochondrial activity. In contrast to NO and AMPK, which could be generated and activated, respectively, in adipocyte tissue in response to acute exercise (Fig. 3), lactate produced in skeletal muscle during exercise circulates through the body, including to adipose tissue (15). Furthermore, adipose tissue also produces lactate (18), and in skeletal muscle cells lactate activates H2O2 production (16). Thus, we posited that lactate and H2O2 are also exercise-inducible factors in adipose tissue during exercise. Although the p-CaMKII/total CaMKII ratio was not altered in response to acute exercise, we attempted to assess the effect of increased intracellular calcium levels. Hence, we determined whether those exercise-inducible factors could activate lipolysis in 3T3-L1 adipocytes. Glycerol release and FA release into the media during lipolytic stimulation were measured for cells treated with either 10 μM or 1 mM H2O2, 10 mM sodium-lactate, 5 mM caffeine, 2 mM AICAR, or 100 μM SNAP. Treatments with sodium-lactate, caffeine, or SNAP each significantly increased glycerol release (Fig. 4), which represents total lipolytic activity in adipocytes (because glycerol produced during lipolysis is not efficiently phosphorylated and used for TAG synthesis due to the poor activity of glycerol kinase in adipocytes) (21, 29). On the other hand, 1 mM H2O2, caffeine, and AICAR each significantly increased FA release (Fig. 4).

Effects of putative exercise-inducible factors on mitochondrial biogenic signaling. We next examined whether exercise-inducible factors could increase mitochondrial biogenic signaling in 3T3-L1 adipocytes. Because 10 μM H2O2 failed to have an effect on lipolytic activity (Fig. 4), we examined the effect of 1 mM H2O2 for the H2O2 treatment. Sodium-lactate (\( P < 0.01 \)) and SNAP (\( P < 0.05 \)) each significantly increased PGC-1α expression, and 1 mM H2O2 significantly increased COx expression (\( P < 0.05 \)) (Fig. 5). H2O2, caffeine, or AICAR did not increase PGC-1α expression (\( P = 0.08, 0.06, \) and 0.18, respectively); and sodium-lactate, caffeine, AICAR, and SNAP did not increase COx expression (\( P = 0.10, 0.24, 0.21, \) and 0.08, respectively).
Effects of putative exercise-inducible factors on LD-associated proteins. We examined whether exercise-inducible factors could increase the expression of LD-associated proteins in 3T3-L1 adipocytes. \( \text{H}_2\text{O}_2 \), sodium-lactate, caffeine, and SNAP each significantly increased HSL expression; and \( \text{H}_2\text{O}_2 \), sodium-lactate, caffeine, and AICAR each significantly increased ATGL expression (Fig. 6). Furthermore, sodium-lactate and SNAP each significantly increased perilipin expression, whereas \( \text{H}_2\text{O}_2 \), sodium-lactate, caffeine, AICAR, and SNAP each significantly increased CGI-58 expression (Fig. 6).

DISCUSSION

The primary novel findings of the present study are that prolonged endurance exercise training increased the expression of LD-associated proteins and mitochondrial biogenic signaling in adipose tissue of DIO rats. Furthermore, exercise-inducible factors such as lactate, ROS, calcium signals, AMPK, and NO, some of which could be generated in adipose tissue, and some of which could be transported from skeletal muscle, were able to enhance lipolytic activity in differentiated 3T3-L1 adipocytes.

Previous studies have demonstrated that exercise training enhanced lipolytic activity in adipose tissues in rats (7, 27), hamsters (2), overweight men (5), obese postmenopausal women (44), or obese women with polycystic ovary syndrome (25), and several potential mechanisms underlying the augmentation of adipocyte lipolysis during exercise have been proposed. For example, Campbell et al. suggested that exercise training increases lipolytic activity through the augmented activity of glucocorticoids concomitant with increased levels of two major lipolytic enzymes, HSL and ATGL, in adipose tissue (2). Ogasawara et al. suggested that exercise training enhances lipolytic activity through elevated levels of ATGL in adipocytes, concomitant with lower levels of plasma insulin (27). Furthermore, Sutherland et al. suggested that exercise training increased mitochondrial biogenesis in rat adipose tissue through an increase in circulating catecholamine levels (36). In the present study, we found that exercise training significantly increased, or tended to increase, levels of perilipin or CGI-58, respectively, in addition to increased HSL and ATGL in epididymal fat tissue. Perilipin and CGI-58 are crucial for the activity of the major lipolytic enzymes HSL and ATGL in adipocytes. Depending on the phosphorylation status, perilipin blocks HSL in basal condition or recruits HSL to LD surfaces in response to PKA stimulation, and reversibly docks CGI-58, an ATGL activator, and the resulting ATGL/CGI-58 complex efficiently hydrolyzes TAG to diacylglycerol and FA (17). Actually, perilipin expression in adipocytes is decreased against DIO (26). Furthermore, perilipin-null mice showed elevated basal lipolysis but decreased catecholamine-stimulated lipolysis, and increased insulin resistance (37). On the other hand, perilipin overexpressed in mice protected against DIO (24). Taken together, the increased expression levels of LD-associated proteins in epididymal fat may be one potential mechanism for the exercise-induced weight loss in DIO rats in the present study, through the augmented capacity of lipolysis.

We also examined levels of mitochondrial biogenic signaling in white adipose tissue in response to exercise training. Although it was not statistically significant, expression of PGC-1α in the exercise training group was more than twice that in the SED control group. Furthermore, COX protein expression was significantly increased by exercise training. As mentioned above, we explored putative factors induced by exercise training to activate mitochondrial biogenic signaling and found that lactate, ROS, and NO increased the levels of mitochondrial biogenic signaling. Thus, lactate, ROS, and NO might be potential mechanisms for augmented mitochondrial biogenic signaling in white adipose tissue in addition to circu-
lating catecholamine (36). These results can be interpreted to mean that exercise training could protect against mitochondrial maladaptations observed in adipose tissue of insulin resistance and T2DM (4, 40). A limitation of the study is that we did not examine the assumption that augmented mitochondrial oxidative activity in white adipocytes may be required to provide energy and cyclic AMP for PKA-mediated lipolysis. However, it is suggested that mitochondrial ATP synthesis is essential for major metabolic pathways including lipolysis in mature adipocytes [see review in (8)]. In line with this, the higher activity of the TAG/FA cycle (i.e., lipolysis/re-esterification) in human visceral white adipose tissue compared with subcutaneous white adipose tissue [see review in (8)]. We consider elevated levels of mitochondrial biogenic signaling, and hence, elevated oxidative activity in white adipose tissue to be supportive of major metabolic pathways including lipolysis. Another limitation of the study is that we solely analyzed epididymal adipose tissue. However, as noted previously [see (8)], there are known metabolic differences between fat pads (33, 39). The extent to which these metabolic responses to exercise-inducible factors are achieved in fat pads other than epididymal fat warrants further study.

Next, we examined whether putative exercise-inducible factors known to be generated in skeletal muscle also could be increased in adipose tissue. Although we could not test whether exercise-inducible factors traveled from muscle to adipose tissue or were generated in adipose tissue, we found that NO production and AMPK activity may be increased by a single bout of exercise in adipose tissue. This interpretation is supported by a previous study demonstrating that AMPK was activated during lipolysis in adipocytes due to an increase in the AMP/ATP ratio (11). As well, Ruderman et al. demonstrated that AMPK activity was increased in adipose tissue 30 min following treadmill running exercise in normal rats (30), suggesting that AMPK is an exercise-inducible factor in adipocytes.

To explore putative factors induced by exercise training to activate lipolysis, we also examined the effects of compounds (such as H2O2, sodium-lactate, caffeine, AICAR, and the NO donor SNAP) that are known to reproduce some of the exercise

![Fig. 3. p-NOS/eNOS ratio, p-AMPK/total AMPK ratio, and p-CaMKII/total CaMKII ratio were examined in adipose tissue of the sedentary control group (CON, n = 6) and acute exercise group (AEX, n = 6) in response to a single bout of exercise. p-NOS/eNOS and p-AMPK/total AMPK ratios in the AEX group were significantly higher than those of the CON group. Representative immunoblots of p-NOS, eNOS, p-AMPK, total AMPK (t-AMPK), p-CaMKII, and total CaMKII (t-CaMKII) are shown. *P < 0.05 vs. CON group.](image)

![Fig. 4. Release of (A) glycerol and (B) fatty acids (FA) into the media upon lipolytic stimulation were measured in differentiated 3T3-L1 cells (n = 6). Treatments with sodium-lactate, caffeine or SNAP significantly increased glycerol release compared with that of the control group. Treatments with 1 mM H2O2, caffeine, or AICAR significantly increased FA release compared with the control group. *P < 0.05, **P < 0.01 vs. control group.](image)
effects in skeletal muscle on levels of LD-associated proteins and mitochondrial biogenic signaling. Among them, lactate, ROS, and NO increased levels of mitochondrial biogenic signaling. Nevertheless, all treatments examined in the present study increased lipolytic activity assessed by glycerol release and/or FA release into media in response to lipolytic stimulation. Interestingly, these treatments increased the expression of LD-associated proteins such as perilipin, CGI-58, HSL, and ATGL, which regulate lipolysis in a coordinated manner. So far, few studies have investigated the physiological factors that increase the expression of LD-associated proteins in adipocytes. For instance, Wohlers and Spangenburg demonstrated that ovariectomy elevated the interaction of CGI-58 and ATGL, and decreased perilipin protein content, whereas supplementation with 17β-estradiol prevented these protein-protein interaction and the reduction of protein content in the visceral fat of rats (42). Gaidhu et al. demonstrated that activating AMPK by AICAR treatment increased the expression of ATGL, whereas it decreased HSL activity (9). Ogasawara et al. suggested that the exercise-induced increase in ATGL expression is regulated through transcriptional activation of the peroxisome proliferator-activated receptor-γ2 (PPARg2) (27). The present study provides new insights into mechanisms of the regulation of LD-associated proteins.

Lactate increased the expression of PGC-1α and LD-associated proteins such as HSL, ATGL, perilipin, and CGI-58, all of which might be potential mechanisms for the increased lipolysis induced by lactate treatment in this study. Also, a previous report indicated that lactate activates GPR81, an orphan G-protein-coupled receptor highly expressed in fat, and suppresses lipolysis in adipocytes (22). In the present study, however, cells were incubated with sodium-lactate for 6 h and

**Fig. 5.** Expression of mitochondria-related proteins such as PGC-1α and COx were examined in differentiated 3T3-L1 cells that were pretreated with 10 mM sodium-lactate, 5 mM caffeine, 2 mM AICAR, 100 μM SNAP for 6 h, or 1 mM H2O2 for 15 min (n = 6). Treatments with sodium-lactate or SNAP significantly increased expression of PGC-1α compared with that of the control group. Treatment with H2O2 significantly increased expression of COx compared with that of the control group. *P < 0.05, **P < 0.01 vs. control group.

**Fig. 6.** Expression of the LD-associated proteins such as perilipin, CGI-58, HSL, and ATGL were examined in differentiated 3T3-L1 cells that were pretreated with 10 mM sodium-lactate, 5 mM caffeine, 2 mM AICAR, 100 μM SNAP for 6 h, or 1 mM H2O2 for 15 min (n = 6). Treatments with H2O2, sodium-lactate, caffeine, or SNAP significantly increased expression of HSL compared with that of the control group. Treatments with H2O2, sodium-lactate, caffeine, or AICAR significantly increased expression of ATGL compared with that of the control group. Treatments with sodium-lactate or SNAP significantly increased expression of perilipin compared with that of the control group. Treatments with H2O2, sodium-lactate, caffeine, or SNAP significantly increased expression of CGI-58 compared with that of the control group. *P < 0.05, **P < 0.01 vs. control group.
then incubated with fresh media without sodium-lactate for 18 h, followed by lipolytic activation. Therefore, lipolytic activity was evaluated in the absence of sodium-lactate. Although precise mechanisms are not certain, the present study suggests that one bout of lactate treatment is capable of increasing lipolysis by means of increased levels of mitochondrial biogenic signaling and expression of LD-associated proteins. In our previous study in L6 myotubes, H$_2$O$_2$ was involved in the regulation of gene expression induced by lactate treatment (16). Although the increased protein expressions in response to H$_2$O$_2$ treatment were not completely the same as lactate-induced protein expressions, H$_2$O$_2$ treatment was also capable of increasing lipolysis conceivably by means of increased mitochondrial biogenesis and LD-associated proteins.

AMPK activation by AICAR treatment profoundly increased FA release, whereas it did not increase glycerol release, which was different from the increased lipolysis induced by lactate or NO-donor SNAP treatments. The increased FA release by AICAR treatment is consistent with our previous study, which also showed that during lipolysis, a significant amount of FAs generated by TAG hydrolysis are re-esterified, leading to formation of micro-LDs (17). As suggested in the previous study (9), AICAR treatment might have reduced re-esterification of FAs in the present study. We found that NO-donor SNAP treatments significantly increased glycerol release, which is contrary to the previous studies in which isoproterenol-stimulated lipolysis was inhibited by SNAP treatments (10, 19). The difference between the present study and previous studies is the way that lipolytic activity was assessed: previous studies assessed lipolytic activity in the presence of SNAP in the media, which might have decreased cAMP production following catecholamine stimulation by interacting with β-adrenergic receptor and/or G protein-coupled receptors (10). Again, similar to the effect of sodium-lactate, increased lipolytic activity induced by SNAP treatment may not be a direct effect of NO, but may due to elevated mitochondrial biogenesis and LD-associated proteins.

In summary, we examined for the first time the effects of exercise-inducible factors such as ROS (H$_2$O$_2$), lactate-inducible signals (sodium-lactate), increased cellular calcium levels (caffeine), AMPK (AICAR), and NO (SNAP) on the expression of LD-associated proteins and mitochondrial biogenic signaling to assess putative factors induced by exercise training to activate lipolysis in differentiated 3T3-L1 adipocytes. Interestingly, lipolytic activity was increased by exercise-inducible factors examined in this study in accordance with elevated levels of LD-associated proteins and mitochondrial biogenic signaling. It should be noted that these exercise-inducible factors could also increase mitochondrial biogenesis in skeletal muscle cells, thereby enhancing the capacity of FA oxidation. Thus these exercise-inducible factors are attractive therapeutic effectors against LD-associated metabolic diseases through activation of lipolysis in adipocytes and of FA oxidation in muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.H. conception and design of research; T.H. and K.S. performed experiments; T.H. analyzed data; T.H. interpreted results of experiments; T.H. prepared figures; T.H. drafted manuscript; T.H. edited and revised manuscript; T.H., K.S., and M.I. approved final version of manuscript.

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