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Hormone-sensitive lipase is critical mediators of acute exercise-induced regulation of lipolysis in rat adipocytes

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ABSTRACT

The purpose of the present study was to investigate the effect of acute exercise on lipolysis via coordination of hormone-sensitive lipase (HSL) and scaffold proteins, i.e., perilipin A and comparative gene identification-58 (CGI-58), in rat primary adipocytes. Glycerol release was significantly elevated immediately (0 h) and three hours (3 h) after exercise. Both activity and localization to the pellet of HSL were significantly greater in the pellet fraction, which is included in lipid droplet associated-proteins, than in the supernatant fraction. In the pellet fraction, although neither perilipin A nor CGI-58 protein level changed, level of perilipin A/CGI-58 complex was significantly reduced, accompanied by up-regulated association of perilipin A/HSL at 0 h and 3 h after exercise. On the other hand, there were no changes in these molecules at 24 h after exercise, despite a significant decrease in lipolysis that was observed in response to isoproterenol. These findings suggest that acute exercise enhances lipolysis up to at least 3 h after exercise in a manner dependent on modification of HSL and its association with and alteration in scaffold protein.

1. Introduction

Physical exercise has been shown to increase plasma catecholamine levels, which in turn, stimulates hydrolysis of lipid droplets in adipocytes to release free fatty acids and glycerol that provide fuel for metabolism. It has been widely accepted that activation of cAMP-dependent protein kinase A (PKA) due to accumulation of intracellular cAMP is the major signaling mechanism by which hormonal stimulation of lipolysis takes place in adipocytes via β_1-, β_2-, and β_3-adrenergic receptor (AR)-mediated signal transduction, which is referred to as the lipolytic cascade [1]. Furthermore, there is growing evidence that both perilipin A and comparative gene identification-58 (CGI-58) protein act as scaffold proteins on lipid droplets in adipocytes. It has been reported that CGI-58 rapidly disperses into the cytoplasm after stimulation of isoproterenol, a selective β_1-, β_2-, and β_3-AR agonist [2], in order to facilitate access of HSL to lipid droplets following activation of PKA, although it binds to perilipin A on lipid droplets under basal conditions [3]. In addition, a study of perilipin A null mice has also demonstrated that hormonal stimulation of lipolysis is significantly less in adipocytes [4], suggesting that dynamic changes in scaffold function of both perilipin A and CGI-58 proteins play important roles in powerful lipolytic machinery in adipocytes.

We have previously demonstrated that intracellular cAMP production in rat epididymal adipocytes is significantly increased immediately (0 h) and at three hours (3 h), but is significantly reduced at 24 h after acute exercise in response to agonist [5]. Moreover, our previous findings have shown that expression of the number of cell-surface β_2-AR is closely associated with changes in intracellular cAMP levels [6], suggesting that acute exercise is capable of regulating physiological and biochemical changes in the lipolytic cascade in adipocytes after exercise. Indeed, it has been shown that cAMP-PKA-mediated phosphorylation of HSL at both Ser 563 and 660 in adipose tissue is modified by the acute exercise [7], and that transient hormonal stimulation of adipocytes leads to alteration in the localization of HSL from cytosol to lipid droplet [8] with quantitative shift of the HSL in PKA-dependent manner [9,10]. To date, however, there is no direct evidence to support the effect of acute exercise on lipolysis in primary adipocytes via change in interaction of HSL with scaffold proteins following trafficking event of HSL.

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Here we demonstrate that elevated lipolysis in adipocytes takes place up to at least 3 h after exercise, accompanied by increases in localization of activated HSL on lipid droplets, and that association of the perilipin A/HSL complex is significantly increased, although association of perilipin A/CGI-58 is significantly decreased at 0 h and 3 h after exercise.

2. Materials and methods

2.1. Animal care and acute exercise program

Male Wistar rats (SLC, Shizuoka, Japan) with initial body weights of 230–260 g were housed two or three to a cage in a temperature-controlled room at 23 °C with a 12:12-h light–dark cycle. Food and water were available *ad libitum*. The animals were randomly divided into four groups – a control group and three acutely exercised groups: immediately after exercise (0 h group), 3 h after exercise (3 h group), and 24 h after exercise (24 h group). After exercise periods, food and water were available *ad libitum*. However, there was no change in food amount of intake between before or after acute exercise at 24 h after exercise group. At these designated time points, our previous study showed that significant changes in intracellular cAMP production in adipocytes [5,6]. The rats in the exercise groups were subjected to exercise on a treadmill set at a 5° incline at 17 m/min for 60 min. This working intensity and duration was approximately 50% VO_2 max, which was estimated based on published data [12,13]. The sedentary control animals were not subjected to running on a treadmill. Thereafter, the rats were sacrificed by cervical vertebra dislocation; the epididymal adipose tissues were removed and used for adipocyte isolation. All experiments conducted in this study were approved by the Animal Care Committee of Tokyo Metropolitan University Graduate School of Science.

2.2. Preparation of adipocytes

Adipocytes were isolated by a modification of the method of Rodbell [14]. Briefly, fat pads were minced with scissors and placed in plastic vials in buffer A (Krebs-Ringer bicarbonate solution buffered with 10 mM HEPES, pH 7.4, containing 5.5 mM glucose and 2% (w/v) fatty acid-free bovine serum albumin) with 200 nM adenosine and collagenase type1 (1 mg/ml, Worthington Biochemical, Lakewood, NJ). Collagenase digestion was performed at 37 °C in a water bath shaker. After 15 min, the contents of the vials were immediately filtered in mesh and centrifuged at 100*g* for 1 min. The layer of floating cells was then washed three times with buffer A. Adipocytes were incubated in plastic vials in a total volume of 500 μl buffer A containing adenosine deaminase (0.05 mg/ml, Sigma, St. Louis, MO). After a 2 min pre-incubation, adipocytes were incubated for 30 min again with or without 50 nM isoproterenol (ISO) to investigate lipolytic responses; the cell-free incubation medium was removed and assayed for glycerol release as an index of lipolysis. The average number of adipocytes and glycerol contents were determined according to the method described earlier [11].

2.3. Adipocyte subcellular fraction

Adipocyte subcellular fractions were prepared by a commercially available kit (ProteoExtract Subcellular Proteome Extraction Kit, EMD Biosciences, Inc., Democrat Road, Gibbstown, NJ) according to the manufacture's protocol. Briefly, adipocytes were collected and washed three times with phosphate-buffered saline; thereafter, cells were homogenized in an ice-cold extraction buffer 1, including both protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma, St. Louis, MO), by 20 passages through a 5/8-inch, 27-gauge needle attached to a syringe maintained at 4 °C for 10 min with gentle agitation. The homogenate was centrifuged at 1000*g* at 4 °C for 10 min. The supernatant obtained was used as the supernatant fraction. The extraction buffer 2, including both protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma, St. Louis, MO), was immediately added to the resultant pellet. The cell pellet was incubated at 4 °C for 30 min with gentle agitation. Following centrifugation at 6000*g* at 4 °C for 10 min, the pellet protein extract obtained by this procedure was used as a pellet fraction. The samples were frozen at −80 °C until use.

2.4. Measurement of HSL activity

Measurement of HSL activity was performed according to the method in the previous study [15]. Briefly, triolein (50 mg) and [^{3}H] triolein (220 μCi) were suspended in 3.75 ml of 5% (w/v) gum Arabic solution and sonicated at 4 °C for 5 min. The assay medium was the following composition in a total volume of 100 μl: 50 μl subcellular enzyme solution, 0.14 μmol of triolein, 0.45 mg of gum arabic, 1.43 mg of bovine serum albumin, 8 μmol of KCl, 2 μmol of NaCl, and 4.5 μmol of *N*,*N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid. After 30 min incubation at 37 °C at pH 6.8, the released [^{3}H] oleic acid was measured by a previously reported method [16].

2.5. Immunoblot analysis

The fractionated samples were used for immunoblotting. There was no significant difference in the protein level between cells from the different groups in each fraction (data not shown). Therefore, identical loading amounts of each sample were run on the same gel. The samples were mixed with Laemmli's sample buffer and then placed in a heat block at 100 °C for 3 min. The samples were cooled and then loaded onto a 9–12% SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred onto a PVDF sequencing membrane (Millipore Corporation, Billerica, MA). The PVDF membrane was first incubated for 60 min in TBS-T (100 mM of Tris–HCl, pH 7.4, 150 mM of NaCl, and 0.1% Tween 20) containing 5% skim milk. After incubation, the PVDF membrane was incubated with a specific antibody in the TBS-T at 4 °C overnight. The following antibodies were used at a 1:1000 dilution: HSL, CGI-58 (Abcam, Cambridge, UK), phosphorylated-HSL on Ser 563, phosphorylated-HSL on Ser 660 (Cell Signaling Technology, Inc., Danvers, MA), perilipin A (Santa Cruz Biotech. Inc., Santa Cruz, CA). After washing, the membranes were incubated for 60 min with anti-rabbit and anti-goat immunoglobulin G (1:2000 dilution)-conjugated horseradish peroxidase antibody (Dakocytomation, Glostrup, Denmark). The membranes were washed, and the immunoreactive bands were detected using the ECL system (GE Healthcare, Buckinghamshire, UK) by Kodak X-ray film (Kodak, Tokyo, Japan).

2.6. Immunoprecipitation

The pellet fractions were incubated with antibody to perilipin, and the immunocomplexes were then recovered by absorption to protein G-agarose beads (Santa Cruz Biotech. Inc., Santa Cruz, CA). The immunocomplexes were subjected to SDS–PAGE. The detection of the immune reactive bands was done as described above.

2.7. RNA extraction and RT-PCR

RNA extraction and RT-PCR analysis of mRNAs for HSL and β-actin were performed using an RT-PCR system, as previously

described [5,6]. Twenty-four cycles of amplification were carried out for HSL mRNAs and 20 cycles were used for β-actin mRNA. The conditions of each cycle were denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The primers are described below:

HSL: 5′-CATAAGACCCCATTGCCTGC-3′ (sense)
5′-TCTACCACTTTCAGCGTCAC-3′ (antisense)
β-actin: 5′-ACCTGACAGACTACCTCATG-3′ (sense)
5-ACTCATCGTACTCCTGCTTG-3′ (antisense).

2.8. Other determinations

Values represent the means ± S.D. The significance of differences between means was assessed by the Scheffe's test after the analysis of variance had been performed to establish that there were significant differences between the groups. $p < 0.05$ was regarded as significant.

3. Results

3.1. Effect of acute exercise on lipolysis and HSL activity

Our previous study demonstrated that acute exercise provoked significant increases in intracellular cAMP accumulation at 0 h and 3 h after exercise in primary rat adipocytes [5], and that the number of cell-surface β_2-AR increased significantly also at 0 h and 3 h, but decreased significantly 24 h after exercise [6]. These findings suggest the possibility that acute exercise is capable of causing changes in lipolysis via the β_2-AR-cAMP pathway at the designated times, because it has been shown that accumulation of intracellular cAMP induces phosphorylation and activation of HSL through cAMP-dependent phosphorylation of PKA, thereby enhancing glycerol release [1]. As would be expected, the glycerol release was significantly increased in both basal and isoproterenol (ISO)-stimulated conditions at 0 h and 3 h after exercise compared with the non-exercised control group, whereas the response to ISO was significantly decreased at 24 h after exercise (Fig. 1A). On the basis of these findings, we further investigated the HSL activity in both supernatant and pellet fractions, because hormonal stimulation of adipocytes have shown to change in the localization of HSL from cytosol to lipid droplet [8]. Interestingly, HSL activities were significantly reduced at 0 h and 3 h after exercise in supernatant fractions, accompanied by increased HSL activities in the pellet fraction under both basal and ISO-stimulated conditions (Fig. 1B and C). Conversely, there was no significant alteration in HSL activities in both fractions at 24 h after exercise. These results suggest that acute exercise-induced enhancement of lipolysis is regulated via the changes in the localization of activated HSL following alteration in intracellular cAMP levels up to at least 3 h after exercise.

3.2. Effect of acute exercise on modification of HSL

Based on the results shown in Fig. 1, localization and phosphorylation of the HSL protein were reconfirmed by immunoblot analysis. Previous studies have shown that cAMP-mediated activation of HSL is regulated by phosphorylation at both Ser 563 and 660, a regulatory domain of the enzyme [7]. As can be seen in Fig. 2A, protein localization of HSL was transferred from supernatant fractions to the pellet fractions at 0 h and 3 h after exercise, but there was no change at 24 h after exercise compared with control. Under this condition, approximately 45% of the total HSL translocated to the pellet fraction, with 70% of total HSL residing in the pellet fraction at 0 h and 3 h after exercise. Moreover, in the supernatant fraction, while there was no significant change in levels of the

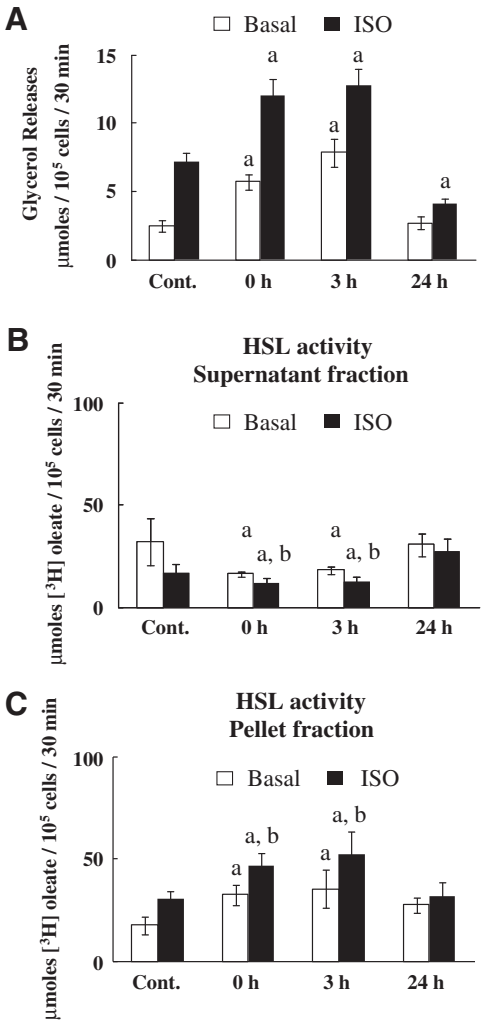

Fig. 1. (A) Effect of acute exercise on the rate of lipolysis in adipocytes with or without isoproterenol (ISO) ($n = 5$). (B and C) Effect of acute exercise on hormone-sensitive lipase activities ($n = 5$). Bars and vertical lines indicate mean ± SD. [a] $p < 0.05$ vs. respective control value of un-exercised group, [b] $p < 0.05$ vs. respective basal value.

phosphorylated-HSL at both Ser site, in the pellet fraction, localization of phosphorylated-HSL, which were normalized to HSL protein, at both Ser 563 and 660 were shown in pellet fractions at 0 h and 3 h after exercise, whereas it returned to the control levels at 24 h after exercise (Fig. 2B and C). In addition, no significant alterations in the level of HSL mRNA were observed at indicated time points (data not shown). These results indicate that acute exercise-induced activation and translocation of HSL is regulated via posttranslational modification event rather than newly synthesized protein of HSL.

3.3. Effect of acute exercise on the expression of perilipin A, CGI-58, perilipin A/CGI-58, and perilipin A/HSL

Both perilipin A and CGI-58 are currently viewed as components of dynamic scaffold proteins that serve as a lipid droplet-associated organizing center for enzymes and transporters involved in lipid metabolism [2,3]. Under basal conditions, although both proteins have been shown to form a complex on the lipid droplet, stimulated lipolysis releases CGI-58 from perilipin A. In turn, perilipin A is capable of binding to phosphorylated-HSL, thereby leading to dramatic enhancement of lipolysis [17]. Hence, we further investigated whether acute exercise contributes to

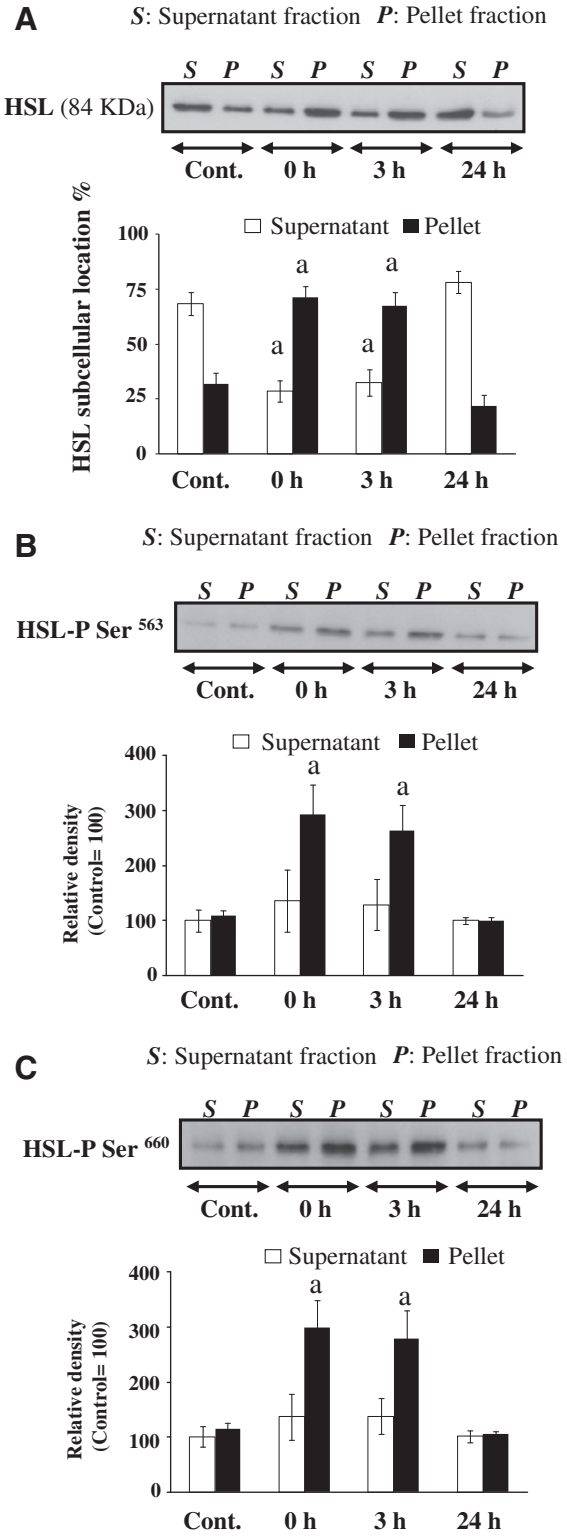

Fig. 2. Effect of acute exercise on levels of HSL protein, phosphorylated-HSL on Ser 563 and 660. Adipocyte subcellular fractions were prepared as described in Section 2. (A, B, and C) Representative immunoblotting data of both supernatant and pellet fractions (upper) and the density relative to the control value (lower) are shown (control = 100; $n = 5$ for each group). Results were representative of three independent experiments. [a] $p < 0.05$ vs. respective control value of un-exercised group.

alterations in the level of perilipin A, CGI-58 and its immunocomplex in the pellet fraction. As shown in Fig. 3A and B, localization of neither perilipin A nor CGI-58 was changed after acute exercise in pellet fraction, whereas association of perilipin A/CGI-58 immunocomplex was significantly reduced at 0 h and 3 h after exercise (Fig. 3C). Moreover, the results of immunoprecipitation showed that the perilipin A/HSL immunocomplex was significantly increased at 0 h and 3 h after exercise (Fig. 3D), suggesting that changes in either association or dissociation actions of scaffold proteins also play a key role in acute exercise-induced increases in lipolysis up to at least 3 h after exercise. In contrast, at 24 h after exercise, there were no significant changes in the localization of perilipin A and CGI-58, and association of both perilipin A/CGI-58 and perilipin A/HSL complexes compared with controls (Fig. 3).

4. Discussion

The current study demonstrated that acute exercise significantly enhanced glycerol releases from rat primary adipocytes at immediately and 3 h after exercise, therein significantly elevated intracellular cAMP levels, via significant increase in association action of perilipin A with HSL following trafficking in HSL from cytosol to lipid droplet, accompanied by dissociation action of perilipin A with CGI-58. This is supported by the following experimental results. In pellet fraction, HSL activity was significantly greater in 0 h and 3 h after exercise than in the non-exercised control, thereby increased in lipolysis. Under these conditions, level of HSL was predominantly observed in pellet fraction, although HSL was widely expressed in supernatant fraction at non-exercised control. Moreover, phosphorylation of HSL at Ser 660, which have been shown to play an important role in trafficking of HSL, was significantly elevated in pellet fraction. Finally, association of perilipin A/HSL complex was augmented at 0 h and 3 h after exercise with dissociation of perilipin A/CGI-58 complex in pellet fraction, suggesting that functional localization and alteration of HSL and scaffold proteins play key roles in regulation of lipolysis in primary adipocytes during and after exercise periods.

It is very interesting to note that acute exercise provokes an increase in either HSL activity or its trafficking from cytosol to lipid droplet at 0 h and 3 h after exercise. Activation of HSL has been shown to be regulated by several molecules, such as AMPK [18], ERK1/2 [19], and PKA [20]. Of these molecules, PKA-dependent phosphorylation of HSL is widely accepted to be due to hormonal activation of HSL, thereby leading to increases in lipolysis [21]. Indeed, several studies have revealed that stimulation of adipocytes with β_1-, β_2-, and β_3-AR agonist leads to changes in the content of HSL protein in subcellular localization in rat adipocytes [22], and that trafficking events are dependent on phosphorylation of HSL at Ser 660 with a significant increase in lipolysis [7,23]. Thus, hormonal stimulation, i.e., by catecholamine, would act as the trigger of both phosphorylation and trafficking of HSL through the β_1-, β_2-, and β_3-AR-cAMP pathway at 0 h after exercise. However, it is conventionally considered that plasma catecholamine levels gradually return to the sedentary control levels for post exercise periods. Therefore, catecholamine-induced stimulation of the β_1-, β_2- and β_3-AR-cAMP would scarcely occur at 3 h after exercise. It may be logical to speculate that, in our previous study, β_2-AR can be kept on the cell surface for at least 3 h after exercise because of down-regulation of the expression of adaptor proteins, which contribute to the internalization of β_2-AR, such as G-protein-coupled receptor kinase-2 and β-arrestin-2 [6]. Thus, the β_2-AR-cAMP pathway might function as a modulator of HSL at 3 h after exercise through up-regulation of β_2-AR. In addition, acute exercise has been shown to down-regulate the expression of Gαi-2 protein, which suppresses intracellular cAMP production following the inhibition of adenylate cyclase activity at 0 h and 3 h after exercise [5]. Thus, a coordinated effect on β_2-AR and G-protein would be associated with a modification of HSL up to 3 h after exercise

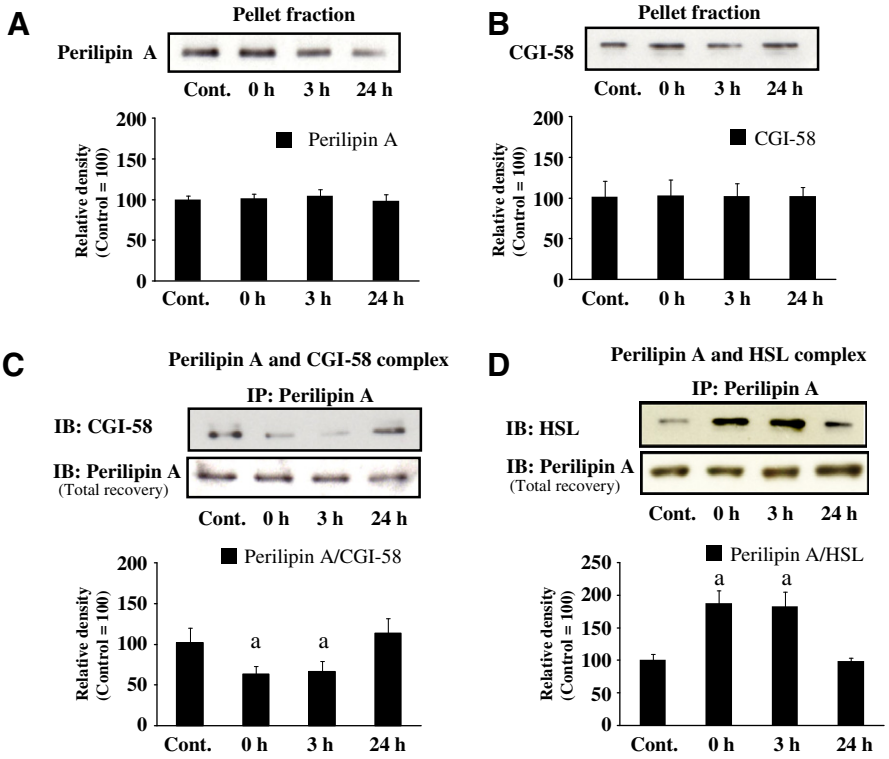

Fig. 3. Effect of acute exercise on the expression of perilipin A, CGI-58, perilipin A/CGI-58 complexes, and perilipin A/HSL complexes in the pellet fraction. (A, B, C, and D) Representative immunoblotting data (upper panel) with the relative density of each band (lower panel) are shown (control = 100; $n = 5$ for each group). (C and D) Representative total recovery blot of perilipin A are shown to reconfirm that same amount of protein are immunoprecipitated. A total of 200 μg of total protein was used for immunoprecipitation. Results were representative of three independent experiments ($n = 5$ for each group). $^{a}p < 0.05$ vs. control value. IB: immunoblot; IP: immunoprecipitation.

through an increase in intracellular cAMP accumulation. On the other hand, at 24 h after exercise, ISO-stimulated lipolysis was significantly reduced relative to ISO-stimulated control cells (Fig. 1A) potentially due to down regulation of β_2-AR on cell surface [6] as basal lipolysis rate between these two groups were not different. We have shown that dobutamine, a selective β_2-AR agonist, induced down-regulation of intracellular cAMP production with reduced expression of β_2-AR mRNA [6], and that expression of Gαi-2 protein significantly increased at 24 h after exercise [5]. Therefore, inhibitory effects on upstream of lipolytic cascade might play a role in the reduction of ISO-stimulated lipolytic response at 24 h after exercise.

It is noteworthy that acute exercise-induced enhancement of lipolysis is closely associated with both dissociation of CGI-58 and association of HSL to perilipin A at 0 h and 3 h after exercise. A previous study has demonstrated that cAMP-PKA-mediated phosphorylation of perilipin A promotes the dissociation of CGI-58 from perilipin A, thereby allowing perilipin A to bind phosphorylated-HSL [17]. In the current study, in the pellet fraction, phosphorylated-HSL was observed at 0 h and 3 h after exercise (Fig. 2), accompanied by a significant increase in association of perilipin A/HSL (Fig. 3D), suggesting that acute exercise-induced functional alterations in perilipin A and CGI-58 are also regulated in concert with and possibly by activating cAMP-PKA pathway, because both basal and agonist-stimulated significant increases in intracellular cAMP levels were observed at 0 h and 3 h after exercise [5,6]. On the other hand, there were no changes in expression of perilipin A, CGI-58 or these complexes at 24 h after exercise. Thus, inactivation of the cAMP-PKA pathway might not affect expression of scaffold proteins at 24 h after exercise, because agonist-stimulated cAMP levels were significantly reduced at 24 h after exercise, accompanied by no change in basal levels of cAMP [5,6].

In summary, the results of the current study indicate that acute exercise enhances lipolysis in adipocytes up to at least 3 h after acute exercise, whereas the lipolysis is decreased at 24 h after the exercise in response to the agonist. The former would be explained at least in part due to increase in intracellular cAMP levels through up-regulation of upstream of lipolytic cascade, i.e., enhanced expression of β_2-AR on cell surface and reduced expression of Gαi-2 [5,6], following localization and alteration of HSL activity via increase in coupling to perilipin A with HSL by exercise. The latter might also be due to down regulation of lipolytic cascade, i.e., reduced expression of β_2-AR mRNA and up regulation of Gαi-2 [5,6], rather than functional change between scaffold proteins and HSL.

5. Conclusion

Acute exercise induces enhancement of lipolysis in adipocytes up to at least 3 h after exercise, accompanied by increases in localization of activated HSL on lipid droplets. Moreover, these alterations are associated with significant increase in levels of perilipin A/HSL complex, although association of perilipin A/CGI-58 is significantly decreased at 0 h and 3 h after exercise.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.026.

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