Biochemical and Biophysical Research Communications 464 (2015) 348–353

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Habitual exercise training acts as a physiological stimulator for constant activation of lipolytic enzymes in rat primary white adipocytes

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ARTICLE INFO

Article history:
Received 23 June 2015
Accepted 24 June 2015
Available online 30 June 2015

Keywords:
Hormone-sensitive lipase
Adipose triglyceride lipase
Phosphorylation
Primary white adipocytes
Rat

ABSTRACT

It is widely accepted that lipolysis in adipocytes are regulated through the enzymatic activation of both hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) via their phosphorylation events. Accumulated evidence shows that habitual exercise training (HE) enhances the lipolytic response in primary white adipocytes with changes in the subcellular localization of lipolytic molecules. However, no study has focused on the effect that HE exerts on the phosphorylation of both HSL and ATGL in primary white adipocytes. It has been shown that the translocation of HSL from the cytosol to lipid droplet surfaces requires its phosphorylation at Ser-563. In primary white adipocytes obtained from HE rats, the level of HSL and ATGL proteins was higher than that in primary white adipocytes obtained from sedentary control (SC) rats. In HE rats, the level of phosphorylated ATGL and HSL was also significantly elevated compared with that in SC rats. These differences were confirmed by Phos-tag SDS-PAGE, a technique used to measure the amount of total phosphorylated proteins. Our results suggest that HE can consistently increase the activity of both lipases, thereby enhancing the lipolysis in white fat cells. Thus, HE helps in the prevention and treatment of obesity-related diseases by enhancing the lipolytic capacity.

1. Introduction

Habitual exercise training (HE) helps to maintain low body weight in laboratory animals and humans. Under a regular exercise regimen, lipolytic responses in white adipocytes are upregulated through adaptive changes in a signal transduction system referred to as a lipolytic cascade [1,2]. Physical exercise stimulates lipid metabolism by modifying lipolysis-related molecules. In mammals, the prolonged HE plays a key role in the positive regulation of energy expenditure.

It has been widely accepted that hormone-sensitive lipase (HSL) is a unique rate-limiting enzyme of lipolysis in white fat cells. The translocation of HSL from the cytoplasm to the lipid droplet surface is mediated by its phosphorylation by protein kinase A (PKA) [2,3]. However, this concept is modified by the identification of adipose triglyceride lipase (ATGL) [4]. ATGL catalyses the first step in triacylglycerol (TG) hydrolysis in the adipose tissue; this is accompanied by HSL-regulated hydrolytic degradation of TG in mammals. ATGL initially hydrolyses TG into free fatty acid (FFA), and HSL subsequently hydrolyses diacylglycerol substrate to produce an additional FFA and a monoacylglycerol [2,3]. Moreover, accumulated evidences show that the activation of ATLG is regulated by phosphorylation by PKA at Ser-404 in humans [5] or Ser-406 in rodents [6]. It has been shown that HE upregulates the action of ATGL in addition to HSL in human skeletal muscle [7] with an increase in its phosphorylation [8]. However, at present, no study has focused on the effect that HE exerts on the phosphorylation of ATGL as well as HSL in primary white adipocytes, although white adipocytes has a central role in both ATGL and HSL. The changes observed in the phosphorylation of these two enzymes by HE would help us better understand HE-induced adaptive changes in lipolytic molecules in white adipocytes because our previous study demonstrated that HE provokes behavioural changes in lipolytic molecules, thereby enhancing the lipolysis in adipocytes obtained from HE [9].

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http://dx.doi.org/10.1016/j.bbrc.2015.06.157
0006-291X/

In the present study, we demonstrate that HE brings about a constant increase in the levels of both phosphorylated HSL and ATGL with upregulation of these proteins in primary rat adipocytes. Our results strongly support the opinion that fitness training is imperative for prevention and treatment of obesity-related metabolic syndromes.

2. Materials and methods

2.1. Animal care and exercise training program

Four-week-old male Wistar rats (SLC, Shizuoka, Japan) were housed in groups of 2 or 3 per 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 two groups: SC group (n = 5) and HE group (n = 5). The HE rats were exercised on a treadmill set at a 5-degree incline, 5 days per week for 10 weeks, according to the previously reported protocol [9–11]. The initial training intensity was 15 m/min for 20 min; thereafter, the running speed and duration were progressively increased until, after 6 weeks, the rats ran continuously at 30 m/min for 90 min. The SC rats were not subjected to the treadmill exercise. The HE rats were euthanized 36 h after the last exercise session. The rats were anesthetised with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight; Abbott Labs, Abbott Park, IL). Adipose tissue was rapidly removed and adipocytes were isolated using the methods described below. The Animal Care Committee of the Kyorin University School of Medicine approved the animal protocol following National Institute of Health guidelines (NIH, USA).

2.2. Preparation of primary adipocytes

Adipocytes were isolated using a method developed by Rodbell [12]. 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 type 1 (3 mg/ml, Worthington Biochemical, Lakewood, NJ). Collagenase digestion was performed at 37 °C in a water-bath shaker. After 60 min, the contents of the vials were filtered and centrifuged at 100 × *g* for 1 min. The layer of floating cells was then washed three times with buffer A. The cells were transferred to centrifugation tube, and then the proteins were extracted.

2.3. Protein extraction

Isolated primary adipocytes were washed three times with phosphate-buffered saline (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4). The cells were centrifuged at 800 × *g* at room temperature for 2 min. The packed cells were homogenized in ice-cold homogenization buffer (Pierce, Rockford, IL), including both protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, St Louis, MO), by 20 passages through a 5/8-inch, 27-gauge needle attached to a syringe, at 4 °C. The homogenate was centrifuged at 40,000 × *g* at 4 °C for 30 min. The obtained supernatant was centrifuged again, and the obtained clear sample was used as the cell extract for immunoblotting analysis. The samples were frozen at −80 °C for later analysis.

2.4. Sample preparation for Phos-tag analysis

Phos-tag acrylamide was purchased from NARD Institute Ltd. (Amagasaki, Japan). The cell lysate was dialyzed in the Xpress Micro Dialyzer MD100 system (Funakoshi Co., Ltd., Tokyo, Japan) to remove detergent. Dialyzed samples were mixed with Laemmli's sample buffer and then placed in a heat block at 95 °C for 5 min. The samples were cooled and loaded onto a 6% Phos-tag SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto a PVDF sequencing membrane (Millipore Corporation, Billerica, MA) according to the manufacturer's protocol. Immunoblotting analysis was performed as described below, using HSL and ATGL antibodies. The principle of Phos-tag SDS-PAGE has been described in the paper by Kinoshita et al. [13].

2.5. Immunoblotting analysis

There was no significant difference between the protein levels in the cells from the different groups (data not shown). Therefore, identical 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 cooled samples were 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 antibodies against the following antigens were used at a 1:1000 dilution: HSL, ATGL phosphorylated at Ser-406, β-actin (Abcam, Cambridge, UK), p-ATGL, HSL phosphorylated at Ser-563, and HSL phosphorylated at Ser-660 (Cell Signaling Technology, Inc., Danvers, MA). After washing, the membranes were incubated for 60 min with anti-rabbit or 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. Statistical analysis

Values represent the means ± S.D. The significance of differences between means was assessed using 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.

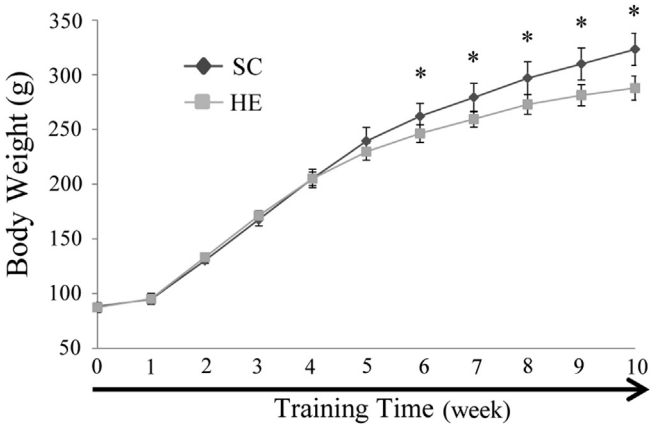

Fig. 1. The effect of habitual exercise training on the alteration in body weight. The body weights were measured by weight scale. HE-induced loss of body weights were observed from six week period of training time. *P < 0.05 vs. sedentary control.

Table 1
Physical characteristics of experimental animals.

	Body weight (g)	Epididymal (g)	Retroperitoneal (g)	Inguinal (g)
Sedentary control (n = 5)	323.2 ± 14.65	5.84 ± 0.49	3.17 ± 0.56	3.12 ± 1.68
Habitual exercise (n = 5)	287.8 ± 11.03*	3.07 ± 0.47*	1.43 ± 0.40*	1.84 ± 0.49*

Values represent the means ± S.D. *P < 0.05 vs. sedentary control.

3. Results

3.1. Physical characteristics of both sedentary control and habitual exercise trained rats

It has reported that the mean body weights are significantly lower in the HE rats than in the SC rats [9,10]. Indeed, induced inhibition of body weight gain was observed from the six week period of training time (Fig. 1). Moreover, masses of the epididymal, retroperitoneal, and inguinal adipose tissue were also significantly decreased in the HE rats compared with the SC rats, respectively (Table 1). These results suggest that isolated adipocytes obtained from HE rats evoke HE-induced adaptive change in cellular energy metabolism, such as enhancement of the lipolytic responses via increase in levels of both HSL and ATGL protein [9].

3.2. HE causes a constant elevation of phosphorylated HSL levels in primary white adipocytes

Hormonal stimulation of white adipocytes activates the intracellular PKA and increases the phosphorylation of HSL. Indeed, the available data demonstrate that site-specific phosphorylation has a critical role in the activation of HSL, resulting in translocation of HSL from the cytosol to lipid droplet surface [3]. Enzymatic activity

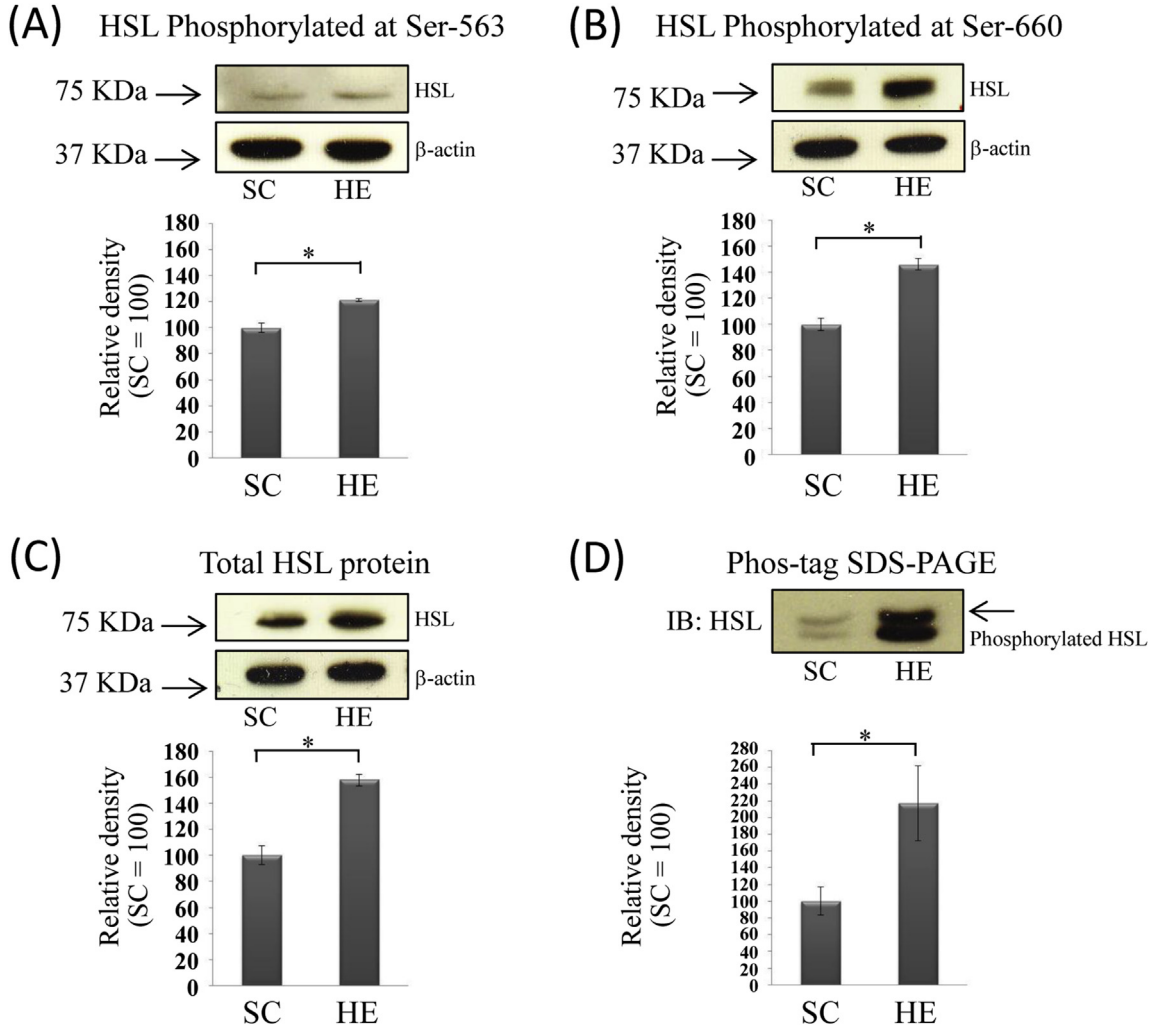

Fig. 2. The effect of habitual exercise training on the levels of phosphorylated HSL and total HSL. Immunoblotting analysis was performed using antibody against HSL Ser-565, HSL Ser-660 and pan HSL. Representative immunoblotting data (upper panel) with the relative density of each band (lower panel) of (A) HSL phosphorylated at Ser-565, (B) HSL phosphorylated at Ser-660, and (C) total HSL protein are shown (sedentary control (SC) = 100, n = 5, each group). (D) Phos-tag SDS-PAGE was performed before immunoblotting analyses are shown. Immunoblotting analysis was performed using antibody against total HSL. Representative immunoblotting data (upper panel) and the relative density (lower panel) of total phosphorylated HSL (control = 100, n = 5, each group). Results are representative of three independent experiments. Bars and vertical lines indicate mean ± SD. *P < 0.05 vs. sedentary control. IB: Immunoblotting.

of HSL is upregulated by phosphorylation of serine residues [14], and the rate of lipolysis in primary white adipocytes is enhanced by HE [9,15]. However, it is not clear whether the levels of phosphorylated HSL in these cells are elevated by HE. As shown in Fig. 2 A and B, we found that the levels of HSL phosphorylated at both Ser-563 and -660, i.e., PKA phosphorylation sites, were significantly higher in the adipocytes obtained from HE than those in SC. The total levels of HSL protein also increased in the HE compared with the SC (Fig. 2C) [9]. These results suggest that HE can lead to a stable increase in the levels of HSL phosphorylation, accompanied by an elevation of HSL protein synthesis. In HE, the increase in levels of phosphorylated HSL (in comparison with SC) was confirmed using Phos-tag SDS-PAGE, a method for detection of total phosphorylated proteins in combination with Western blotting analysis (Fig. 2D). Thus, in primary white adipocytes, HE could improve the ability to respond to lipolytic stimulation by maintaining the elevated levels of phosphorylated HSL.

3.3. HE constantly augments the levels of phosphorylated ATGL in primary white adipocytes

ATGL has a phosphorylation site at Ser-404 in humans and Ser-406 in rodents. These sites are phosphorylated in white adipocytes, during fasting and exercise, by PKA [6]. Notably, ATGL plays a critical role in the first step of TG hydrolysis in the lipid droplets [16], the coordinated action of ATGL and HSL regulates the rate-limiting step of lipolysis in white fat cells. The levels of ATGL protein and its mRNA significantly increase in HE animals in comparison with SC [9]. However, it is not clear whether phosphorylation of ATGL in primary white adipocytes is changed by HE. We found (Fig. 3A and B) that phosphorylation of ATGL at Ser-406 was significantly higher in HE group than that in SC group; this increase was accompanied by elevated levels of ATGL protein. These observations were confirmed by Phos-tag SDS-PAGE (Fig. 3C). Our results suggest that HE-induced phosphorylation of ATGL might also play

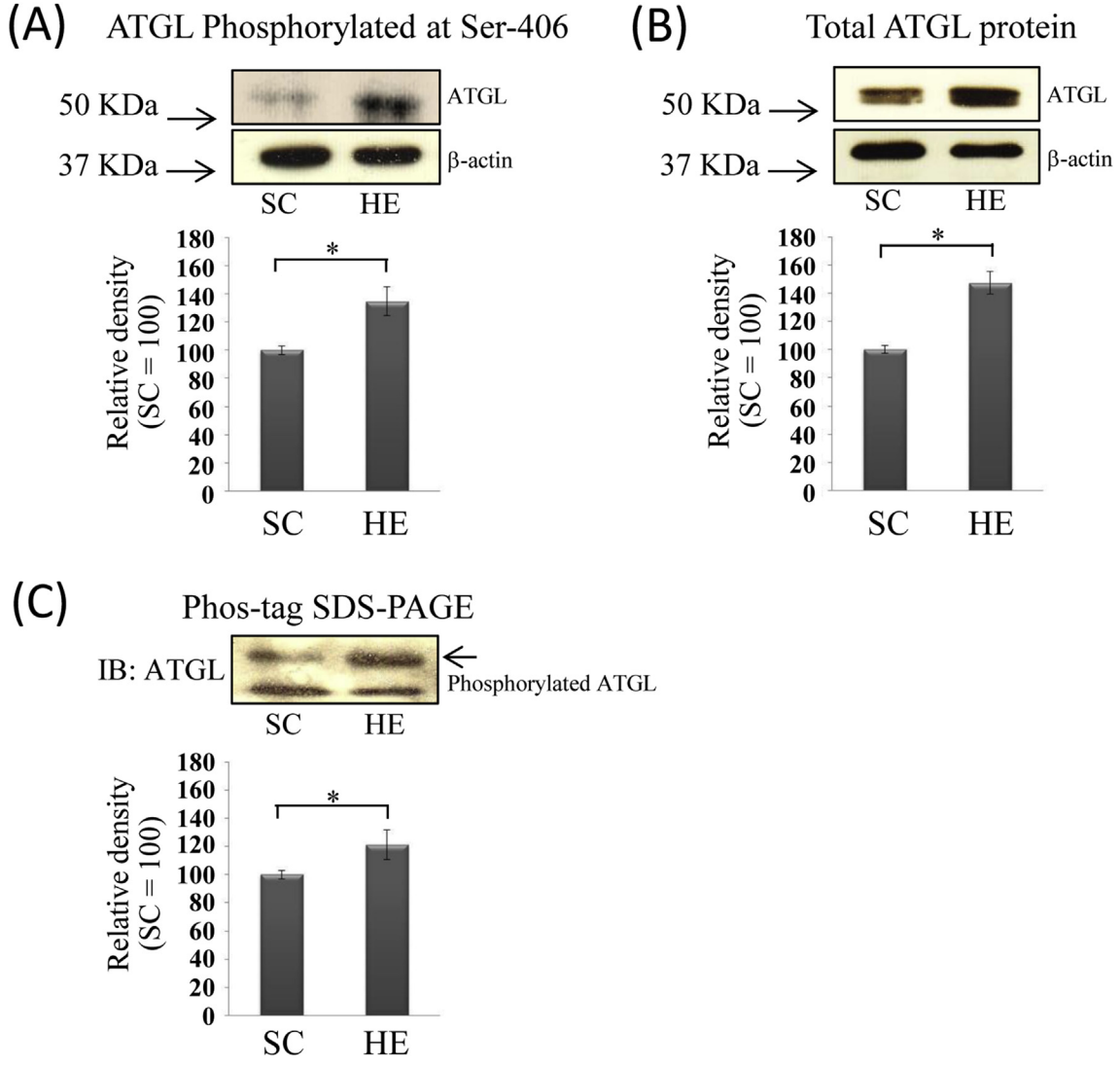

Fig. 3. The effect of habitual exercise training on the levels of phosphorylated ATGL and total ATGL. Immunoblotting analysis was performed using antibody against ATGL Ser-406 and pan ATGL. Representative immunoblotting data (upper panel) with the relative density (lower panel) of (A) ATGL phosphorylated at Ser-406 and (B) total ATGL protein are shown (control = 100, n = 5, each group). (C) Phos-tag SDS-PAGE was performed before immunoblotting analysis. Immunoblotting analysis was performed using anti-ATGL antibody. Representative immunoblotting data (upper panel) and the relative density (lower panel) of total phosphorylated ATGL are shown (control = 100, n = 5, each group). Results are representative of three independent experiments. Bars and vertical lines indicate mean ± SD. *P < 0.05 vs. sedentary control. IB: Immunoblotting.

an important role in constant upregulation of lipolytic response in white adipocytes.

4. Discussion

It has been widely accepted that lipolytic response in white adipocytes is elevated by HE [17–20]. The lipolytic rates of white adipocytes are determined by subcellular localization and/or phosphorylation of lipolytic molecules by PKA [3]. However, the mechanism(s) underlying HE-induced adaptive changes in lipolytic enzyme activity are not well understood. Here we observed an increase in the levels of phosphorylated HSL and ATGL in response to HE (in comparison with SC). These results suggest that HE can accelerate the responses to an upstream molecule, i.e. PKA, in the primary rat white adipocytes. The activity of PKA in white adipocytes has been shown to be significantly increased by HE [15]. In our previous study, we have found that lipolytic enzymes localize on lipid droplets in HE animals, increasing the release of glycerol and FFA from primary white adipocytes under both basal and isoproterenol-stimulation conditions [9]. Therefore, a stable elevation in the levels of phosphorylated HSL and ATGL caused by HE could be a critical mediator of the increase in the rates of lipolytic response in the primary white adipocytes.

Notably, we found that HE caused a significant increase in the levels of ATGL phosphorylated at Ser-406 in comparison with control (Fig. 3B). It has been reported that exercise provokes phosphorylation of ATGL [6], suggesting that phosphorylation of ATGL caused by exercise might be associated with changes in the PKA activity. In our previous study, we have demonstrated that the activity of PKA, the levels of PKA-anchoring protein 150 (AKAP150, anchoring PKA to the substrate) and of HSL protein in the lipid droplet fraction are increased by HE [15]. The contact between PKA and ATGL might also be facilitated through the anchoring action of AKAP150. Consequently, an increase in PKA–ATGL contact might play a key role in the augmentation of phosphorylated ATGL levels by HE in the primary adipocytes. It has been also reported that ATGL activity is enhanced by 5′-AMP-activated protein kinase (AMPK)-mediated phosphorylation at Ser-406 in mice [21]. Exercise increases AMPK activity in human [22] and rat [23] adipocytes, and AMPK activity increases in adipocytes from rats trained for 6 weeks [24]. This elevated activity of AMPK might lead to an increase in the levels of phosphorylated ATGL in primary white adipocytes.

However, it is more difficult to explain the mechanisms underlying the HE-induced changes in phosphorylation of HSL, which is not modified by PKA. HSL has five phosphorylation regions [25]. AMPK and extracellular-signalling regulatory kinase (ERK) phosphorylates HSL at Ser-565 [26] and Ser-600 [27], respectively. AMPK inhibits HSL activity in L6 myotubes and is associated with reduced phosphorylation of HSL Ser-660. However, in 3T3-L1 adipocytes, AMPK activation does not inhibit HSL activity or glycerol release, and HSL phosphorylation at Ser-660 is maintained [22]. HE also affects ERK activity in several tissues [28–30]. Thus it is possible that HE induces phosphorylation of HSL Ser-565 and -600 through ERK and AMPK. This idea is supported by the present findings that marked increase in phosphorylated HSL were observed in adipocytes obtained from HE animals compared with those obtained from SC (Fig. 2D). Thus, a combined effect of several molecules (AMPK, ERK and PKA) might be responsible for the elevated levels of phosphorylated HSL caused by HE.

In conclusion, HE has a stimulatory effect on phosphorylation of both HSL and ATGL, rate-limiting enzymes of lipolysis, in the primary white adipocytes. We have previously demonstrated that HE provokes several adaptive alterations in these cells, such as increase in the lipolytic response [9], reduction in the cell diameter [31] and improvements in the aberrant adipokine secretion from adipocytes [10]. The present study suggests that HE would accelerate the hydrolysis of intracellular TG by the constant increase in the activity of HSL and ATGL caused by phosphorylation, resulting in a sequence of changes described in the previous studies. Our results confirm the importance of regular exercise in prevention and treatment of obesity in mammals.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements and funding

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Ministry of Education, Culture, Sports, Science and Technology (No. 26350819).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.157.

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