

## Effects of nonexhaustive bouts of high-intensity intermittent swimming training on GLUT-4 expression in rat skeletal muscle

Eri Fujimoto · Shuichi Machida · Mitsuru Higuchi ·  
Izumi Tabata

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**Abstract** We previously reported that 14 bouts of exhaustive high-intensity intermittent training [20 s periods of swimming while carrying a weight (14% of body weight), separated by pauses of 10 s] is the highest stimuli in terms of exercise training-induced glucose transporter 4 (GLUT-4) expression in rat epitrochlearis (EPI) muscles. In the present study, we found that the GLUT-4 protein content in the skeletal muscle of male Sprague-Dawley rats (age 5 weeks old; body weight 90–110 g) that underwent intermittent exercise training of 3 and 14 bouts of 20 s swimming for 5 days was increased over age-matched sedentary control rats by 75 and 71%, respectively, 18 h after the last bout of exercise. These results suggest that GLUT-4 content in rat EPI muscle increases dramatically after very short (60 s) and nonexhaustive high-intensity intermittent exercise training.

**Keywords** High-intensity intermittent exercise · GLUT-4 · Rat · Skeletal muscle

### Introduction

Skeletal muscle is responsible for at least 80% of glucose uptake in humans [1], and, under most physiological conditions, glucose transport is the rate-limiting step in skeletal muscle glucose metabolism [2]. Furthermore, maximal insulin- and contraction-stimulated glucose transport activity is reported to be linearly related to the content of the glucose transporter 4 (GLUT-4) isoform of the glucose transporter in muscle [3, 4]. Therefore, the level of GLUT-4 in skeletal muscle may be an important determinant of whole body glucose disposal. It is known that physical training increases GLUT-4 content in skeletal muscle [5–7], while inactivity induces inverse effects [8]. 5' AMP-activated protein kinase (AMPK) activation and elevation of calcium in skeletal muscle during muscle contraction have been postulated as two mechanisms that induce GLUT-4 expression after exercise training [9, 10]. Since both signals are exercise-intensity dependent [11, 12], we hypothesized in our previous study [13] that the higher the exercise intensity, the higher the GLUT-4 expression after exercise training. Consequently, we found that GLUT-4 content after high-intensity intermittent exercise training of short duration (net exercise time: 280 s) is comparable to that induced by low-intensity prolonged (360 min) exercise training, which is regarded as the maximal stimulus related to exercise training [13].

However, since the protocol examined in our previous study is exhaustive and not thought to be suitable for exercise oriented toward health promotion, we observed the dose-dependent effects of different bouts of high-intensity intermittent exercise on GLUT-4 expression in epitrochlearis (EPI) muscle. Consequently, we found that only 3 intermittent bouts of high-intensity exercise training increase GLUT-4 protein content in the muscle to a level

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E. Fujimoto · I. Tabata (✉)  
Health Promotion and Exercise Program, National Institute of  
Health and Nutrition, 1-23-1 Toyama, Shinjuku City,  
Tokyo 162-8636, Japan  
e-mail: tabata@nih.go.jp

E. Fujimoto · M. Higuchi  
Faculty of Sport Sciences, Waseda University,  
135-1 Horinouchi, Tokorozawa City, Saitama 359-1165, Japan

S. Machida  
School of Physical Education, Tokai University,  
1117 Kitakaname, Hiratsuka City, Kanagawa 259-1292, Japan

that is not statistically different from that observed after exhaustive training consisting of 14 bouts of intermittent exercise. These results suggest that nonexhaustive, intermittent exercise is probably safer and more effective in improving glucose metabolism in skeletal muscle.

## Materials and methods

### Materials

All chemicals were purchased from Sigma Chemical (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan).

### Treatment of animals

All rats used for the present investigation were purchased from CLEA Japan (Tokyo, Japan). The animals were housed in rooms lighted from 7 a.m. to 7 p.m. and were maintained on an ad libitum diet of standard chow and water. The room temperature was maintained at 20–22°C. Prior to the swimming exercise and training experiment, all rats were acclimated to swimming exercise for 10 min day<sup>-1</sup> for 2 days, as described by Ren et al. [14]. All animal experiments were conducted with the approval of the National Institute of Health and Nutrition Ethics Committee on Animal Research.

### High-intensity intermittent swimming exercise training

Twenty-four 5-week-old male Sprague-Dawley rats with body weights ranging from 90 to 110 g were randomly assigned to a control group and two exercise training groups [one group undergoing 3 bouts of high-intensity intermittent swimming exercise training (HIT) and one group undergoing 14 bouts of HIT]. During HIT, the rats performed the designated number of 20 s swimming bouts while bearing a weight equivalent to 14 and 15% of their body weight for the first 3 and last 2 days, respectively. A 10 s pause was allowed between exercise sessions. The rats performed the above swimming protocol once a day for 5 days. Each rat performed the swimming exercise alone in a barrel filled to a depth of 25 cm. The water temperature was maintained at 35 ± 1°C during the exercise. Food intake for all rats was restricted to 8 g from 7:00 p.m. on the last day before the experiment. All rats ate all 8 g of food. Approximately 18 h after the last bouts of training exercise, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg 100 g<sup>-1</sup> body weight), and their EPI muscles, which had been shown to be recruited during swimming exercise [15], were dissected

out. Age-matched sedentary control rats were kept in cages until they were sacrificed.

### Measurement of GLUT-4 protein content

Immediately after dissection of the EPI muscles, the muscles were clamp-frozen by tongue, cooled by liquid nitrogen and stored in a deep freezer (-80°C) until analysis. EPI muscles were homogenized in 29 volumes of ice-cold 10 mM HEPES, 1 mM EDTA, 250 mM sucrose, 1 mM NaF, 1 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), and 2 μl ml<sup>-1</sup> Protease Inhibitor Cocktail (Sigma), pH 7.4, buffer. The homogenate thus obtained was centrifuged for 10 min at 13,000×g at 4°C. Protein concentrations were determined by the bicinchoninic acid (BCA) method [16] in triplicate. Aliquots of homogenate (40 μg of protein) were solubilized in Laemmli sample buffer [17], subjected to SDS-PAGE, and electrophoretically transferred to a polyvinylidene difluoride (PVDF) sheet. The sheet was incubated overnight at 4°C with primary monoclonal GLUT-4 antibody (Chemicon, Temecula, CA, USA) diluted 1:5,000 in 5% skimmed milk. After overnight incubation, the sheet was incubated for 1 h at room temperature with anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive bands were detected by ECL plus (GE Healthcare UK, England).

### Measurement of citrate synthase activity

For the enzyme activity measurements, 10% homogenates were made from the EPI muscles in 175 mM KCl, 10 mM GSH, and 2 mM EDTA, pH 7.4. These homogenates were frozen and thawed four times and mixed thoroughly before measurement of the enzyme activities. The citrate synthase activity in the muscle was measured using Srere's method [18].

### Acute bouts of high-intensity intermittent swimming exercise

Twenty-four 5-week-old male Sprague-Dawley rats with body weights ranging from 110 to 130 g were used in the present experiment. The rats performed the high-intensity intermittent swimming exercise (HIE) used for the HIT exercise protocol described previously. Immediately after the exercise, the rats were anesthetized with inhalation of chloroform (diethyl ether) and a subsequent intraperitoneal injection of pentobarbital sodium (5 mg 100 g<sup>-1</sup> body weight). Their EPI muscles were then dissected out within 5 min after cessation of the exercise. Age-matched sedentary control rats were kept in cages until they were sacrificed.

### Measurement of AMPK phosphorylation

The EPI muscles were homogenized with 29 volumes of ice-cold 10 mM HEPES, 1 mM EDTA, 250 mM sucrose, 1 mM NaF, 1 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ), and  $2 \mu\text{l ml}^{-1}$  Protease Inhibitor Cocktail (Sigma), pH 7.4, buffer. The homogenate thus obtained was centrifuged for 10 min at  $13,000\times g$  at  $4^\circ\text{C}$ . Protein concentration was determined by the BCA method [16] in triplicate. Aliquots of homogenate (40  $\mu\text{g}$  of protein) were solubilized in Laemmli sample buffer [17], subjected to SDS-PAGE, and electrophoretically transferred to a PVDF sheet. The sheet was incubated overnight at  $4^\circ\text{C}$  with primary phospho-AMPK- $\alpha$ (Thr172)antibody (Cell Signaling, Beverly, MA, USA) diluted 1:500 in 5% bovine serum albumin (BSA). After overnight incubation, the sheet was incubated for 1 h at room temperature with anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch). Immunoreactive bands were detected by ECL plus (GE Healthcare UK, England). Total AMPK $\alpha$  was also determined using the sheets that had been immunoblotted for phosphorylated AMPK $\alpha$  (Thr172). The sheets were incubated with stripping buffer [62.5 mM Tris-HCl (pH 6.7), 100 mM  $\beta$ -mercaptoethanol, 2% SDS] at  $50^\circ\text{C}$  for 30 min. The sheets were performed with total AMPK $\alpha$  (Cell Signaling) antibody using the protocol described above.

### Measurement of glycogen concentration

Immediately after acute bouts of the high-intensity intermittent exercise, the EPI muscles were dissected and clamp-frozen by tongue, cooled by liquid nitrogen and stored in a deep freezer ( $-80^\circ\text{C}$ ) until analysis. EPI muscles were weighed and homogenized in 0.3 M perchloric acid and then stored for later determination of glycogen concentrations. Glycogen concentrations were determined by enzymatic methods according to Lowry and Passonneau [19] after acid hydrolysis.

### Measurement of lactate concentrations in blood

Immediately after the different bouts of high-intensity exercise, the rats were anesthetized with inhalation of chloroform (diethyl ether) and a subsequent intraperitoneal injection of pentobarbital sodium (5 mg  $100 \text{ g}^{-1}$  body weight), and blood was obtained from the tail vein. Blood samples were analyzed for lactate levels using the YSI 1500 lactate analyzer (YSI Life Sciences, Yellow Springs, OH, USA).

### Measurement of GLUT-4 mRNA

Six and 18 h after the exercise (HIE), the EPI muscles were dissected and clamp-frozen by tongue, cooled by liquid nitrogen and stored in a deep freezer ( $-80^\circ\text{C}$ ) until analysis. Total RNA from the EPI muscle was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Because the RNA extraction procedure required a large amount of tissue, two EPI muscles were homogenized together. The DNase-treated total RNA (1  $\mu\text{g}$ ) was reverse-transcribed (RT) into cDNA by using random primers and ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA). Aliquots of each RT reaction were added to a PCR master mix (Promega) containing *Taq* DNA polymerase, dNTPs,  $\text{MgCl}_2$ , reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR, and 10 pmol of both sense and antisense primers (forward 5'-GTGTGGTCAATACCGTCTTCACG-3', reverse 5'-CCATTTGCCCTCAGTCATTC-3'). The reaction medium was subjected to PCR amplification. After the lid was warmed at  $94^\circ\text{C}$  for 2 min, the PCR mixtures were subjected to a 40-cycle profile, including denaturation for 60 s at  $94^\circ\text{C}$ , hybridization for 60 s at  $57^\circ\text{C}$ , and elongation for 60 s at  $72^\circ\text{C}$ . In the present investigation, 18S rRNA expression was simultaneously measured as an internal standard using a QuantumRNA 18S Internal Standard Kit (Ambion, Austin, TX, USA). The PCR products were separated by electrophoresis on 2% agarose, stained with SYBR Green (Molecular Probes, Eugene, OR, USA), photographed, and analyzed by densitometry (LAS3000, Fujifilm, Tokyo, Japan). The ratio of GLUT-4 to 18S rRNA standard band densities was then calculated.

### Statistical analysis

All values are expressed as mean  $\pm$  SD. Statistical comparisons were made by one-way analysis of variance (ANOVA) using the Jandel SigmaStat statistical software (Jandel, San Rafael, CA, USA). Whenever the ANOVA indicated significant differences, the Tukey test was used for post-hoc analysis. The statistical significance was defined as  $P < 0.05$ .

## Results

Effects of high-intensity intermittent swimming exercise training with different numbers of exercise bouts on the body weight of rats

The body weight rats did not differ among the sedentary control and the two training groups (Table 1).

**Table 1** Effect of high-intensity intermittent swimming training with different numbers of exercise bouts on body weight of rats

	Control	Number of bouts	
		3	14
Body weight (g)	176 ± 8	174 ± 9	175 ± 6

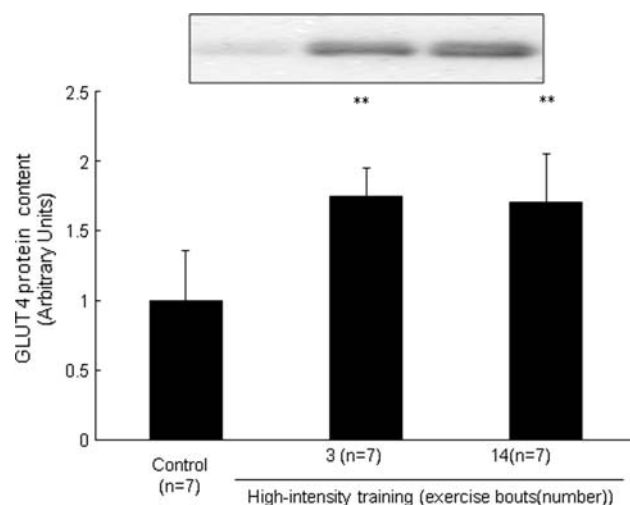
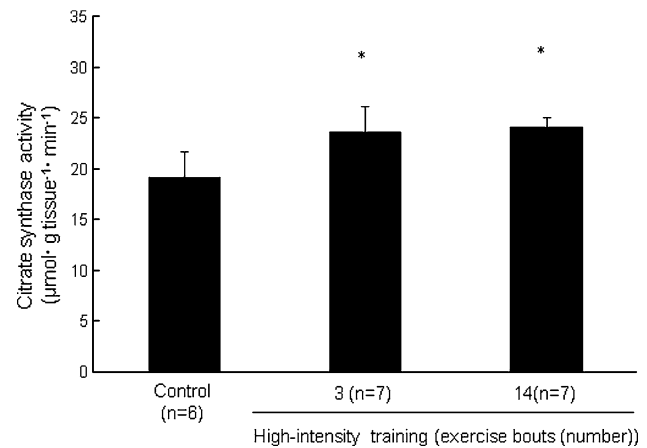
Values are mean ± SD for eight rats

#### Effects of high-intensity intermittent swimming exercise training with different numbers of exercise bouts on GLUT-4 protein in rat EPI muscle

The GLUT-4 protein content in rat EPI muscle was significantly elevated by 75 and 71%, after 3 and 14 bouts of exercise training, respectively, compared with that of the age-matched sedentary control rats ( $P < 0.01$ ; Fig. 1). There was no difference in muscle GLUT-4 between the two training groups.

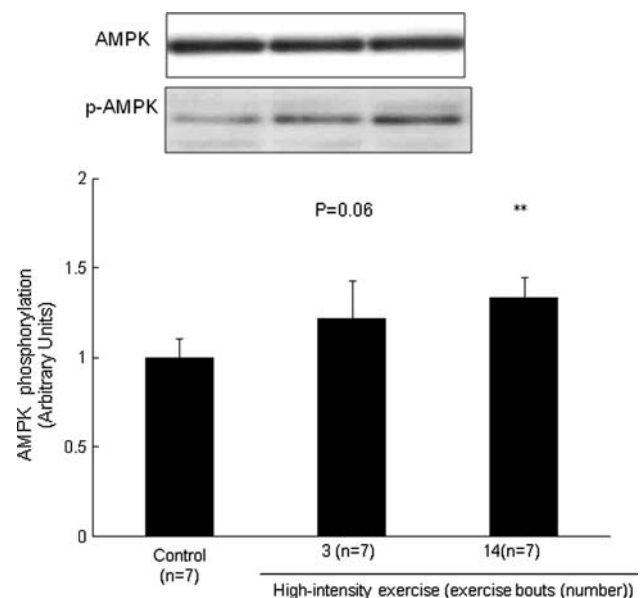
#### Effects of high-intensity intermittent swimming exercise training with different numbers of exercise bouts on citrate synthase activity in rat EPI muscle

Citrate synthase activity in rat EPI muscle after the training with 3 and 14 bouts of exercise was significantly higher than that in the same muscle of the control rats by 23 and 25%, respectively, 18 h after the last bouts of training ( $P < 0.05$ ; Fig. 2). No significant difference in citrate synthase activity was observed among the two training groups with different numbers of high-intensity exercise bouts.

**Fig. 1** Effects of high-intensity intermittent swimming training with different numbers of exercise bouts on GLUT-4 protein content in rat EPI muscle.  $**P < 0.01$  compared with the control group. Values are mean ± SD for seven muscles**Fig. 2** Effects of high-intensity intermittent swimming training with different numbers of exercise bouts on citrate synthase activity in rat EPI muscle.  $*P < 0.05$  compared with the control group. Values are mean ± SD for six to seven muscles

#### Effects of acute high-intensity intermittent swimming exercise with different numbers of exercise bouts on AMPK phosphorylation in rat EPI muscle

AMP-activated protein kinase phosphorylation in rat EPI muscle was significantly elevated by 33% after 14 bouts of exercise, compared with that of the age-matched sedentary control rats ( $P < 0.05$ ; Fig. 3). However, the  $P$  value was near significance level between the controls and those undergoing three bouts of exercise ( $P = 0.06$ ).

**Fig. 3** Effects of high-intensity intermittent swimming with different numbers of exercise bouts on AMPK phosphorylation in rat EPI muscle.  $**P < 0.01$  compared with the control group.  $P = 0.06$  indicates a nonsignificant difference between the group undergoing three bouts of exercise and the control group. Values are mean ± SD for seven muscles

**Table 2** Effect of high-intensity intermittent swimming exercise (HIE) with different numbers of exercise bouts on blood lactate concentration in rats

	Control	Number of bouts	
		3	14
Lactate concentration (mmol l <sup>-1</sup> )	2.5 ± 0.7	6.8 ± 1.8**	14.4 ± 2.9***, †††

Values are mean ± SD for five to eight rats

\*\**P* < 0.01 and \*\*\**P* < 0.001 compared with the control group

††† *P* < 0.001 compared with the group experiencing three HIE bouts

Effects of acute high-intensity intermittent swimming exercise with different numbers of exercise bouts on blood lactate concentrations

The blood lactate concentrations were significantly elevated after 3 (from 2.5 to 6.8 mmol l<sup>-1</sup>; *P* < 0.01) and 14 (from 2.5 to 14.4 mmol l<sup>-1</sup>; *P* < 0.001) bouts of exercise compared with those of control rats (Table 2). Blood lactate concentrations after 3 bouts of exercise were significantly lower than those observed after 14 bouts of exercise (*P* < 0.001; Table 2).

Effects of acute high-intensity intermittent swimming exercise with different numbers of exercise bouts on glycogen concentrations in rat EPI muscle

The glycogen concentrations in rat EPI muscle were significantly reduced by 44% (*P* < 0.01) and 79% (*P* < 0.001) after 3 and 14 bouts of the high-intensity intermittent exercise, respectively, compared with those of the nonexercise rats (Table 3). Glycogen concentrations after 14 bouts of exercise were 66% lower than the levels observed after 3 bouts of exercise (*P* < 0.05; Table 3).

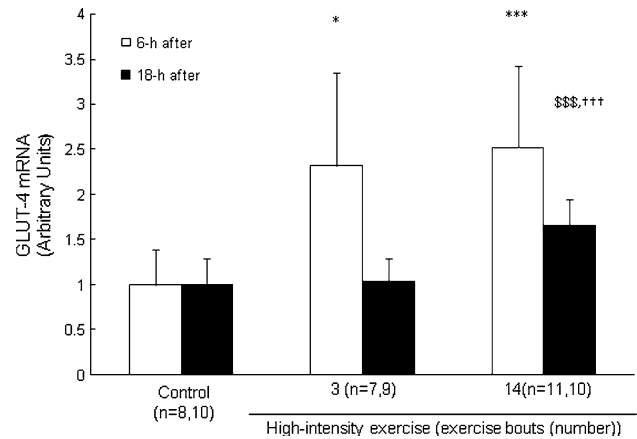
**Table 3** Effect of high-intensity intermittent swimming exercise with different numbers of exercise bouts on glycogen concentration in rat EPI muscle

	Control	Number of bouts	
		3	14
Glycogen concentration (μmol g muscle <sup>-1</sup> )	25.6 ± 2.9	14.4 ± 5.8**	5.5 ± 3.6***, †

Values are mean ± SD for five to seven muscle samples

\*\**P* < 0.01 and \*\*\**P* < 0.001 compared with the control group

† *P* < 0.05 compared with group experiencing three HIE bouts



**Fig. 4** Effect of acute bouts of high-intensity intermittent swimming exercise with different numbers of exercise bouts on GLUT-4 mRNA in rat EPI muscle. \**P* < 0.05 and \*\*\**P* < 0.001 compared with the control group at 6 h. <sup>SSS</sup>*P* < 0.001 compared with the control group at 18 h. †††*P* < 0.001 compared with the group undergoing three bouts of high intensity intermittent swimming exercise (at 18 h). Values are mean ± SD for 7 to 11 muscles

Effects of acute high-intensity intermittent swimming exercise with different numbers of exercise bouts on GLUT-4 mRNA in rat EPI muscle

The GLUT-4 mRNA in rat EPI muscle was significantly increased by 132% (*P* < 0.05) and 152% (*P* < 0.001) 6 h after 3 and 14 bouts of exercise, respectively, compared with that of the aged matched sedentary control rats (Fig. 4). Eighteen hours after the 14 bouts of exercise, the GLUT-4 mRNA still remained at a level higher than the sedentary control by 65% (*P* < 0.001; Fig. 4). There was no statistically significant difference in GLUT-4 mRNA between sedentary control rats and exercised rats that had performed three bouts of the intermittent exercise at the time.

Discussion

The present investigation demonstrated that exercise training consisting of only three nonexhaustive bouts (20 s × 3 = 60 s) of extremely high-intensity intermittent swimming exercise elevates GLUT-4 protein in rat EPI muscle to levels comparable to those attained after 14 exhaustive bouts of exercise training, which has been regarded as the maximal exercise-related stimulus.

From a physiological perspective, the overall effect of exercise training on recruited muscle during a specific type of exercise training generally depends on the exercise intensity and exercise time (number of bouts). Because maximal exercise time is dependent on the exercise intensity, we previously [13] compared changes in GLUT-4 after low- and high-intensity training with exercise



during which the rat becomes virtually exhausted. In the previous investigation, a stint of 14 bouts of high-intensity exercise training, as was used for the present investigation, was adopted to compare the effects on GLUT-4 concentration with those induced by low-intensity exercise training (for 6 h/day). It was found that the high-intensity exhaustive intermittent training increased GLUT-4 content to the same levels as those observed after the low-intensity exhaustive training that had been regarded as the maximal stimulus to GLUT-4 expression in rat skeletal muscle.

Since the HIT protocol (8–10 exhausting bouts of 20 s high-intensity exercise with 10 s rest between bouts) adopted in that study was originally developed for elite athletes [20], the protocol is exhaustive, as indicated by the high blood lactate concentrations (11.4 mM [13]), and not thought to be suitable for exercise prescriptions oriented toward health promotion. Therefore, for the purposes of finding the suitable number of high-intensity intermittent training bouts to elevate GLUT-4 concentrations to satisfactory levels, as compared to the levels attained with exhaustive training, we observed the effects of nonexhaustive high-intensity intermittent exercise (three bouts) on GLUT-4 expression in EPI muscle. Consequently, we found that a shorter training period with only three bouts of the same intensity exercise increases GLUT-4 expression to levels comparable to those obtained with high-intensity intermittent training that results in the rats becoming exhausted every day. Since blood lactate concentrations after 3 bouts of exercise were not as high as those observed after 14 bouts of exercise (Table 2), the training is not considered to be exhaustive. Furthermore, the muscle glycogen content after 3 bouts of the high-intensity exercise was not as low as that observed after 14 bouts of exercise (Table 3). Therefore, short-term high-intensity exercise training might be an effective tool for not only experimental animals but also for healthy humans in terms of preventing diabetes safely by increasing the GLUT-4 protein content in skeletal muscle.

The present investigation has demonstrated that exercise training consisting of only three nonexhaustive bouts ( $20\text{ s} \times 3 = 60\text{ s}$ ) of extremely high-intensity intermittent swimming exercise elevates GLUT-4 protein in rat EPI muscle to a level comparable to that attained after 14 exhaustive bouts of exercise training, which has been regarded as the maximal exercise-related stimulus to GLUT-4 expression (Fig. 1). These results suggest that the effects of high-intensity training on GLUT-4 expression become saturated after a limited number of bouts. This finding might be explained by two different mechanisms. One is that with the shorter exercise training period, the GLUT-4 expression machinery, possibly including pathways of transcription and translation of the protein, cannot respond to higher levels of exercise-related signal(s),

depending on the number of high-intensity exercise bouts. The other possibility is that exercise-related signal(s) related to GLUT-4 expression have already peaked after three bouts of high-intensity exercise.

The present investigation and previous mechanism-oriented studies might imply the latter, which might be explained by the following rationale. First, AMPK phosphorylation, which has been postulated as an exercise-related signal of exercise-induced GLUT-4 expression in skeletal muscle, is known to be dependent on exercise intensity [11, 21] and exercise duration (and number of exercise bouts) [22]. Therefore, it is assumed that the higher the exercise intensity or the greater the number of exercise bouts, the higher the levels of AMPK phosphorylation and GLUT-4 expression after exercise training. However, the present investigation showed that both GLUT-4 expression after high-intensity training with an increased number of exercise bouts and AMPK phosphorylation after the same bouts of exercise used in the training tended to reach a plateau after three bouts of the exercise protocol (Fig. 3). These results might suggest that at least one signal (AMPK phosphorylation) peaked after the third bout of exercise training and that GLUT-4 protein was expressed according to the signal (AMPK phosphorylation).

Since GLUT-4 protein content is at least partially controlled by transcriptional regulation, mRNA of GLUT-4 presumably increases after any kind of training exercise that elevates the GLUT-4 protein content in recruited skeletal muscle. In the present investigation, GLUT-4 mRNA in rat EPI muscle was significantly elevated 6 h after the 3 bouts of HIE to levels comparable to those attained after 14 exhaustive bouts of HIE (Fig. 4). Since the magnitude of the increase may depend on the exercise stimuli related especially to exercise intensity, it is noteworthy that the mRNA levels of GLUT-4 had increased at 6 h after the HIE to the same level as in the rats that had undergone what is believed to be the maximal stimulus in terms of exercise-induced GLUT-4 protein expression.

Further, GLUT-4 mRNA after the high-intensity exercise with 14 bouts remained higher than that of the sedentary rats, whereas the mRNA of the rats undergoing 3 bouts returned to levels similar to those of the control rats 18 h after the exercise. Therefore, for unknown reasons, stimulation of GLUT-4 mRNA expression after 14 bouts of high-intensity exercise appears higher than that induced by 3 bouts of the same exercise. However, since there was no statistical difference in GLUT-4 content 6 h after the training, these results might suggest that elevation of GLUT-4 mRNA for at least 6 h after three bouts of high-intensity exercise is saturated in terms of inducing GLUT-4 protein after high-intensity exercise in rat skeletal muscle.

Since GLUT-4 and mitochondrial enzymes are often expressed simultaneously, and expression of the two

functional proteins is hypothesized to be regulated by the same mechanism [23], we measured citrate synthase activity in the same muscle from the training rats. Consistent with the expression of GLUT-4 protein, we observed that only three nonexhaustive bouts (20 s  $\times$  3 = 60 s) of HIT elevated the activity of citrate synthase in rat EPI muscle to a level comparable to that attained after 14 exhaustive bouts of exercise training at the same intensity (Fig. 2).

In conclusion, the present investigation has demonstrated that only three bouts (net exercise time: 60 s) of high-intensity intermittent exercise training increases GLUT-4 content to the maximal level attained with exhaustive high-intensity intermittent exercise in rat skeletal muscle, which has been regarded as the maximal stimuli in terms of exercise-protocol for GLUT-4 expression.

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