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Glycogen overload by postexercise insulin administration abolished the exercise-induced increase in GLUT4 protein

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Summary

To elucidate the role of muscle glycogen storage on regulation of GLUT4 protein expression and whole-body glucose tolerance, muscle glycogen level was manipulated by exercise and insulin administration. Sixty Sprague-Dawley rats were evenly separated into three groups: control (CON), immediately after exercise (EX0), and 16 h after exercise (EX16). Rats from each group were further divided into two groups: saline- and insulin-injected. The 2-day exercise protocol consisted of 2 bouts of 3-h swimming with 45-min rest for each day, which effectively depleted glycogen in both red gastrocnemius (RG) and plantaris muscles. EX0 rats were sacrificed immediately after the last bout of exercise on second day. CON and EX16 rats were intubated with 1 g/kg glucose solution following exercise and recovery for 16 h before muscle tissue collection. Insulin (0.5 μU/kg) or saline was injected daily at the time when glucose was intubated. Insulin injection elevated muscle glycogen levels substantially in both muscles above saline-injected group at CON and EX16. With previous day insulin injection, EX0 preserved greater amount of postexercise glycogen above their saline-injected control. In the saline-injected rats, EX16 significantly increased GLUT4 protein level above CON, concurrent with muscle glycogen supercompensation. Insulin injection for EX16 rats significantly enhanced muscle glycogen level above their saline-injected control, but the increases in muscle GLUT4 protein and whole-body glucose tolerance were attenuated. In conclusion, the new finding of the study was that glycogen overload by postexercise insulin administration significantly abolished the exercise-induced increases in GLUT4 protein and glucose tolerance.

Introduction

During intensive muscular work, muscle glycogen becomes the major fuel supporting ATP resynthesis [1]. Bergstrom and his colleagues [2, 3] had reported that recovery following a glycogen-depleting exercise results in an increase in muscle glycogen to a higher level than is found in the preexercise state, termed "glycogen supercompensation." During this recovery period, increasing demand on carbohydrate substrate by skeletal muscle is associated with the improvement in the whole-body glucose tolerance [4, 5].

Glycogen-depleting exercise is found to increase expression of the proteins that govern

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glucose uptake in skeletal muscle, including GLUT4 and mitochondrial enzyme [4, 6]. Since skeletal muscle is the major tissue for postprandial glucose disposal, this molecular adaptation could account for the improvement in insulin sensitivity in normal individuals and diabetes patients [7]. Evidence from transgenic mice with glucose transporter protein overexpressed in skeletal muscle provides a direct causal link between the capability of glycogen storage and insulin-dependent glucose transport process [8, 9]. Additionally, exercise training normally elevates muscle citrate synthase (CS) activity (a mitochondrial marker), suggesting that capability of glucose oxidation is also increased by exercise [4], and takes part for the improvement in glucose tolerance.

Several lines of evidence suggest that a negative feedback mechanism might exist for regulating glucose uptake in skeletal muscle by the amount of glycogen storage. Kawanaka et al. [10] reported that the insulin-stimulated signaling pathway for muscle glucose transport is impaired in glycogen-supercompensated muscles. Furthermore, carbohydrate supplementation is found to abolish the rapid increase in GLUT4 mRNA of exercised muscle [6, 11]. It is currently unknown whether glycogen overload by insulin treatment has effect on the whole body glucose tolerance, GLUT4 protein level, and CS activity. Therefore, the main purpose of the study was to determine the interactive effect of exogenous insulin administration and exercise on glucose tolerance, and its association with muscle glycogen, GLUT4 protein level, and CS activity following a 16-h recovery period.

Materials and methods

Experimental animals

Sixty male Sprague-Dawley rats from the National Animal Laboratory of the NSC (National Science Council, Taipei, Taiwan, ROC) weighing 200 g each were provided normal rat chow (PMI Nutrition International, Brentwood, MO, USA) and water *ad libitum*. The temperature of the animal room was maintained at 23 °C, with a artificial 12-h light–dark cycle. After 1 week of housing familiarization, the rats were weight-matched and evenly separated into three groups: control (CON), immediately after exercise (EX0), and 16 h after exercise (EX16). Rats from each group were either intraperitoneal (IP)-injected with human insulin (0.5 μU/kg, Eli Lilly and Company, Indianapolis, Indiana, USA) or with saline ($n = 10$ for each group). The 2-d exercise protocol consisted of 2 bout of 3-h swimming with 45-min rest for each day. The temperature of the water was maintained at 34 ± 1 °C; three rats were placed in each barrel at the same time of swimming.

For the first day of treatments, all rats were intubated with 1 g/kg glucose solution immediately after exercise or insulin treatments for 16 h recovery, while rat chow and water were accessible *ad libitum*. On the second day, same amount of glucose was provided for CON and EX16 rats immediately after exercise and insulin treatments for 16 h recovery until muscle tissue collection, whereas EX0 rats were sacrificed immediately after exercise for muscle tissue collection. Rat chow was provided only for the first 6 h for recovery following exercise or insulin treatment. During the exercise period, food for all rats was removed. RG and plantaris muscles were excised and examined for glycogen content, GLUT4 protein level, and CS activity.

Oral glucose tolerance test (OGTT)

A separate group of rats ($n = 60$) with the same experimental design was used for determining glucose tolerance. The levels of fasted and postprandial glucose concentration were measured for each group. All animals were fasted for 10 h prior to OGTT, which was 16 h following exercise or insulin injection for CON or EX16 groups and was immediately after exercise for EX0 group. The 1 g/kg of glucose (w/v) solution was orally delivered with a stomach tube to rats during the OGTT. Blood samples were taken from the tail at 0 (fasted sample), 30, and 60 min after the oral glucose load for blood glucose and insulin measurements. Postprandial glucose level was the mean glucose concentration of 30 and 60 min after oral glucose challenge. Glucose was measured according to in the method of glucose oxidase as described previously [12].

GLUT4 protein levels

Muscle samples for GLUT4 protein were homogenized in ice-cold HES (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 1 mM EDTA,

and 250 mM sucrose, pH 7.4) buffer (1:20) with a Polytron homogenizer (Kinematica, Littau, Switzerland). Sample homogenates and standards were diluted 1:1 with Laemmli sample buffer (125 mM Tris, 20% glycerol, 2% SDS, and 0.008% bromophenol blue, pH 6.8). The Western blotting procedure for GLUT4 analysis was followed the previously described method [13]. Muscle homogenates containing 25 μg (RG and plantaris muscles) of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to a PVDF membrane. GLUT4 antiserum (Chemicon, Temecula, CA, USA) was used for immunoblotting (directly against the carboxyl-terminus of the GLUT4 protein) in a dilution of 1:5000. GLUT4 protein was visualized using an ECL Western blot detection kit (Amersham, Arlington Heights, IL, USA) on X-ray film according to the manufacturer's instructions.

Glycogen assay

About 50 mg of muscle sample was dissolved in 1 N KOH at 70 °C for 30 min. Dissolved homogenate was neutralized by glacial acetic acid and incubated overnight in acetate buffer (0.3 M sodium acetate, pH 4.8) containing amyloglucosidase. The reaction mixture was neutralized with 1 N NaOH. Samples were then analyzed by measuring glucosyl units using the Trinder reaction (Sigma, St. Louis, MO, USA).

CS activity

CS activity was determined in the plantaris and RG muscles as originally described by Srere [14]. Briefly, samples were homogenized in HES buffer in a 1:40 dilution. The supernatant was assayed spectrophotometrically using DTNB. Assays were performed at 37 °C in a spectrophotometer (Beckman, Fullerton, CA, USA) equipped with a thermoelectric flow cell and a 1-cm light path.

Statistical analysis

A student *t*-test was performed to distinguish significant mean differences between the insulin-injected and their saline-injected control groups for all variables tested. An analysis of variance (ANOVA) among EX0, EX16, and CON groups was performed for all variables. Fisher's protected least significance test, which holds the value of type I errors to 0.05 for each test, was used to distinguish significant differences between pairs of groups. $p < 0.05$ was considered statistically significant. All values are expressed as the mean ± SE.

Results

Time sequence of the exercise and insulin treatments is indicated in Figure 1. For saline-injected rats, the exercise protocol depleted glycogen significantly in both RG (Figure 2a) and plantaris (Figure 2b) muscles. Following 16 h postexercise recovery, glycogen of EX16 in both muscles was increased above CON. For non-exercised rats (CON), insulin administration significantly increased glycogen above the saline control. Immediately after exercise (EX0), insulin-injected rats preserved greater amount of glycogen in both muscles compared to the saline-injected controls. With a 16-h postexercise recovery (EX16), insulin injection significantly elevated the level of glycogen above the saline-injected exercise controls, demonstrating an enhancement in glycogen supercompensation.

Glucose levels under fasted (10 h fasting) and glucose-challenged condition were shown in Table 1. Sixteen hour after insulin injection, fasted glucose level of CON and EX0 was significantly elevated above their saline-injected control. No significant difference was found between the insulin- and saline-injected groups in fasted glucose level at EX16. The postprandial glucose was significantly greater in the insulin-injected group than those in the saline-injected group at CON and EX16. No significant difference was found between the insulin- and saline-injected groups at EX0. For both saline- and insulin-injected rats, fasted glucose level of EX0 was significantly lower than that of CON. For the insulin-injected rats, postprandial glucose level of EX0 was significantly lower than that of CON, and postprandial glucose level of EX16 was significantly greater than that of the CON.

In the saline-injected rats, exercise with 16 h recovery significantly elevated GLUT4 protein level in both RG (Figure 3a) and plantaris (Figure 3b) muscles. Insulin injection did not significantly affect

GLUT4 protein level in the CON and EX0 rats. With 16 h postexercise recovery, insulin administration significantly attenuated the exercise-induced increase in GLUT4 protein. CS activity in both muscles was not significantly affected by either insulin or exercise treatment (Figure 4).

Discussion

Exercise with 16-h recovery significantly increased glycogen level above the non-exercised control in skeletal muscle. This result demonstrates that the current exercise-recovery regimen effectively produces glycogen supercompensation. Since under postprandial condition glucose transport process is the rate-limiting step for glycogen storage [8, 15] and GLUT4 protein is the predominant isoform of glucose transporter expressed in skeletal muscle, the glycogen supercompensation phenomenon is apparently associated with the exercise-induced increase in GLUT4 protein. More importantly, the postexercise insulin administration significantly enhances the level of glycogen supercompensation

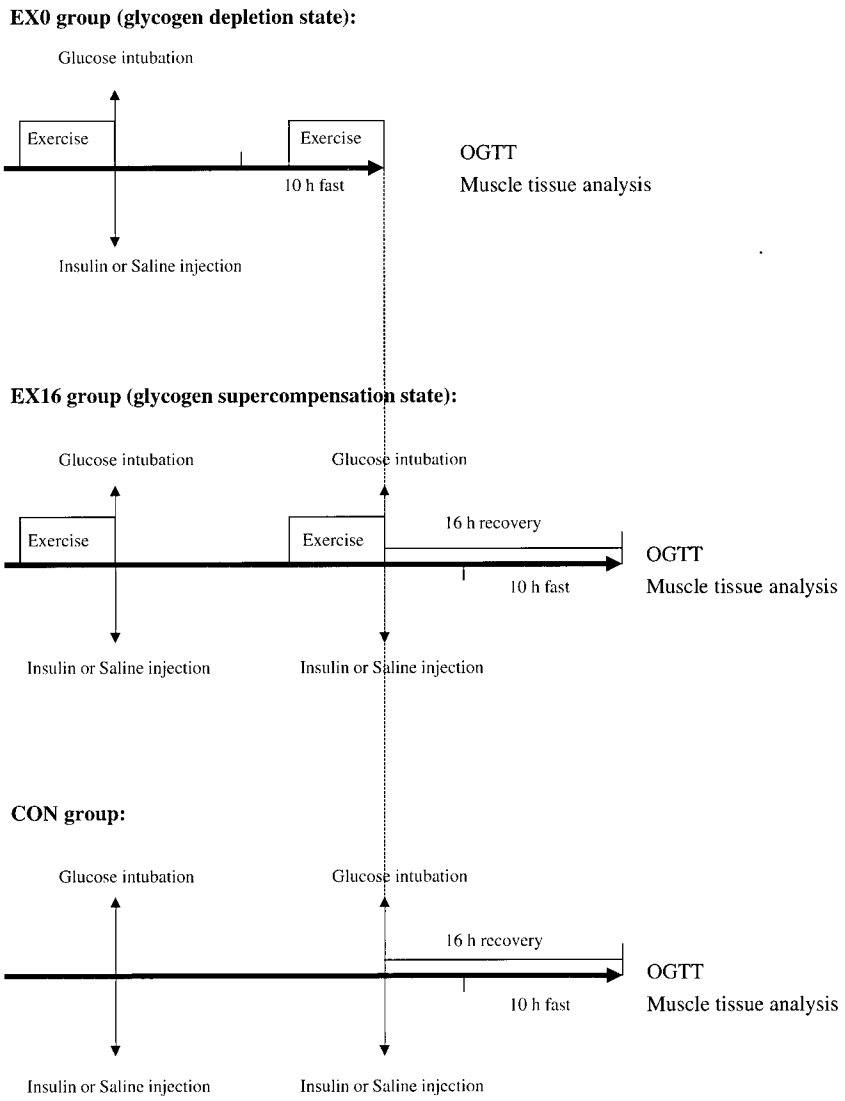

Figure 1. Time sequence for insulin administration and exercise. EX0 ($n=10$ for saline-injected; $n=10$ for insulin-injected), EX16 ($n=10$ for saline-injected; $n=10$ for insulin-injected), and CON ($n=10$ for saline-injected; $n=10$ for insulin-injected) represent immediately after exercise, 16 h recovery after exercise, and sedentary control, respectively.

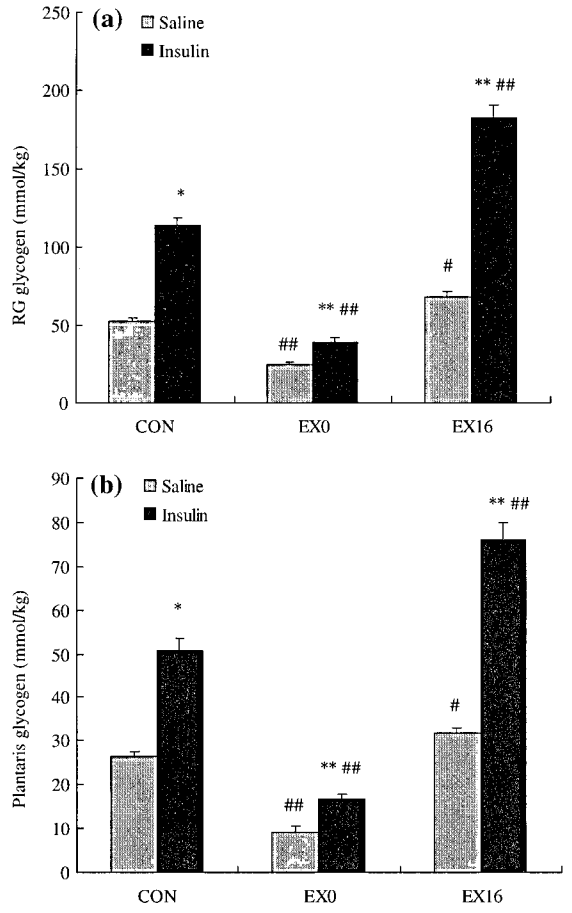

Figure 2. Effect of insulin administration and exercise on glycogen levels in RG (a) and plantaris (b) muscles. 16 h prior to muscle tissue collection, all rats were orally intubated with 1 g per kg of 50% W/V glucose solution. Rat chow was provided for the first 6 h of recovery. Significant difference between insulin- and saline-injected group at * $p < 0.05$ and ** $p < 0.01$. Significant difference against CON at # $p < 0.05$ and ## $p < 0.01$.

above the saline-injected EX16 rats. Under this condition, the GLUT4 protein level was substantially downregulated towards CON level. This novel finding suggests that glycogen overload elicited a negative feedback mechanism by regulating glucose transport system.

The current study also indicates that the amount of glycogen level in skeletal muscle play an important role for regulating the whole-body glucose tolerance. Glycogen depletion by exercise generates the lowest fasted and postprandial glucose levels demonstrating an improvement in glucose tolerance. However, this exercise-induced benefit was significantly attenuated in the glycogen-overloaded rats. It is normally thought that the normal improvement in glucose tolerance by exercise is associated with increase in muscle GLUT4 protein expression [4]. In this study we provide additional knowledge that when muscle glycogen is increased above normal level, GLUT4 protein level becoming independent of glucose tolerance. This result suggests that regular glycogen depleting exercise is essential to maintain optimal whole-body glycemia before glycogen is refilled.

The ability to maintain glucose homeostasis after carbohydrate ingestion relies on the efficient glucose metabolism in skeletal muscle, resulting from parallel expression of the proteins controlling glucose uptake and disposal, including glucose transporter and mitochondrial enzymes for glucose oxidation. Therefore, alteration in the oxidative capacity of a muscle may also contribute a change in glucose disposal property. In the present study, exercise and insulin did not significantly alter CS activity (a mitochondria marker), indicating that the attenuation in glucose tolerance in the glycogen-overloaded rats was less likely due to the change in muscle glucose oxidation capability. Torgan et al. [16] has previously found that exercise training simultaneously elevates the level of GLUT4 protein and CS activity. Apparently, the 2-d exercise protocol is

Table 1. Fasting and postprandial glucose concentration.

	CON (mg/dl)		EX0 (mg/dl)		EX16 (mg/dl)	
	Fasted	Postprandial	Fasted	Postprandial	Fasted	Postprandial
Saline	77 ± 3.8	129 ± 6.4	60 ± 4.3#	109 ± 5.2	85 ± 4.1	139 ± 5.9
Insulin	89 ± 4.1	155 ± 7.5	79 ± 3.9#	119 ± 5.3	91 ± 4.1	168 ± 7.3#
α	0.017	0.006	0.001	0.092	0.144	0.002

α represents probability of type I error for comparing mean difference between insulin- and saline-injected groups. Significant difference against CON at # $p < 0.05$.

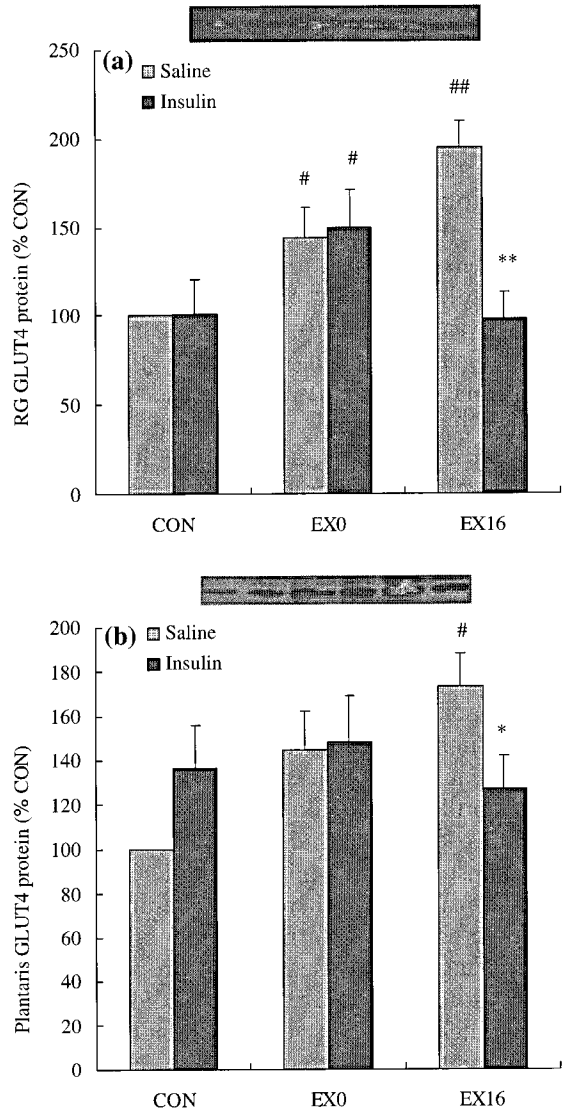

Figure 3. Effect of insulin administration and exercise on GLUT4 protein levels in RG (a) and plantaris (b) muscles. 16 h prior to muscle tissue collection, all rats were orally intubated with 1 g per kg of 50% W/V glucose solution. Rat chow was provided for the first 6 h of recovery. Significant difference between insulin- and saline-injected groups at * $p < 0.05$ and ** $p < 0.01$. Significant difference against CON at # $p < 0.05$ and ## $p < 0.01$. Representative autoradiogram bands showing labeled GLUT4 protein appear above appropriate column for each treatment group.

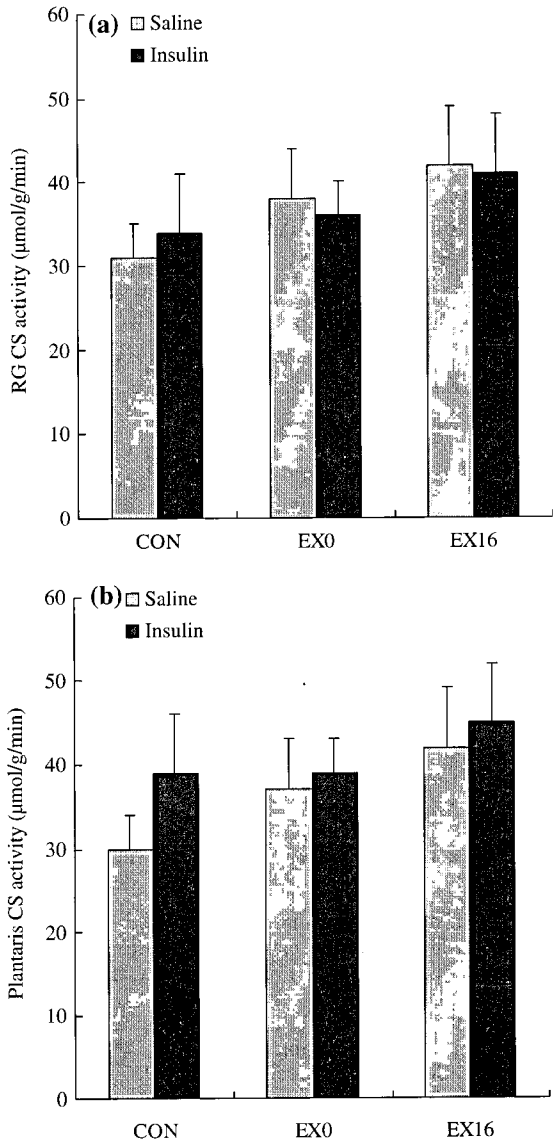

Figure 4. Effect of insulin administration and exercise on CS activity in RG (a) and plantaris (b) muscles.

insufficient to produce equivalent increase in mitochondria biogenesis as did by chronic exercise training. This result also suggests that the early improvement in glucose tolerance by exercise is independent of the change in capability of muscle glucose oxidation.

Previous studies in perfused hindlimb [17] and isolated muscle [18] demonstrate that insulin accelerates muscle glucose transport by translocating GLUT4 protein to plasma membrane. As a result, the rate of glucose uptake increases in skeletal muscle. In this study, we further showed that exogenous insulin administration in the whole animal effectively elevated glycogen storage in RG and plantaris muscles. Under this condition, the total amount of GLUT4 protein in both muscles was not significantly changed. Therefore, the

observed increase in glycogen storage is most likely explained by greater translocation of GLUT4 protein from intracellular pool to plasma membrane, which in turn accelerates the glucose supply for glycogen synthesis [17].

Several lines of evidence from previous studies also support our hypothesis that the glucose disposal *in vivo* is regulated in a negative feedback fashion by amount of glycogen storage level. First, an early study by Fell et al. [19] using dietary manipulation after exercise showed that the rate of glucose uptake in isolated muscle was significantly higher (60–80%) at the same insulin concentration in animals in which muscle glycogen was preserved lower than in those in which glycogen was elevated by carbohydrate supplementation. Second, we previously found that exercise induces increase in GLUT4 protein level within 5 h, but GLUT4 mRNA was rapidly downregulated to baseline as the muscle glycogen returns to normal in rats [6] and human [11]. But unlike the result of the current study, the downregulation of GLUT4 protein has not yet been observed in saline-injected EX16 rats, suggesting that the glycogen level must high enough to elicit the negative feedback mechanism. Third, Garcia-Roves et al. [20] has found that the increases in GLUT4 protein and increased capacity for glycogen supercompensation persisted unchanged for more than 2 day in rats fed a carbohydrate-free diet that prevented glycogen supercompensation after exercise. The result of the current study provides an additional insight into how skeletal muscle prevents glycogen overload.

Unlike fat as a major fuel for mammals, glycogen with its anaerobic nature confers an advantage for encountering acute stress when oxygen demand is suddenly elevated leading to tissue hypoxia. However, a great number of hydroxyl-group on glycogen makes this molecule heavier due to greater association to water by hydrogen bonding. Therefore, excess amount of glycogen storage may not necessarily be advantageous for mobility to an animal not persistently encountering emergency situation 24 h a day. Carrying greater weight could result in higher resting energy consumption for an animal. In this regard, the existence of negative feedback regulation ensuring optimal amount of glycogen storage in skeletal muscle by regulating GLUT4 protein expression appears to be more functionally realistic for animal pursuing efficient mobility. Additionally, this study also highlights an importance value of glycogen depletion on maintaining normal glucose tolerance. This aspect might be evolutionally important since glycogen depletion is a normal lifestyle for a living animal struggling survival by daily muscular work. Reduced physical activity due to fewer requirements in muscular work could lead to high glycogen storage in skeletal muscle, which in turn attenuates glucose tolerance.

Conclusion

This study demonstrates, for the first time, that postexercise glycogen overload by exogenous insulin administration caused an inhibitory effect on normal exercise training-induced increase in GLUT4 protein expression in skeletal muscle. This result suggests that a negative feedback mechanism by suppressing glucose transporter system was operated to prevent muscle glycogen overload. In addition, glycogen overload significantly reversed the exercise effect on improving glucose tolerance.

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