THE EFFECT OF INSULIN AND CARBOHYDRATE SUPPLEMENTATION ON GLYCOGEN REPLENISHMENT AMONG DIFFERENT HINDLIMB MUSCLES IN RATS FOLLOWING PROLONGED SWIMMING

AUTHORS: Lin C.-H.[1], Liao Y.-H.[2], Kuo C.-H.[3], Hou C.-W.[3], Chen C.-Y.[3], Hsu M.-C.[4]

[1] Physcial Education Office, Yuan Ze University, Taoyuan, Taiwan
[2] Exercise Physiology and Metabolism Laboratory, Department of Kinesiology and Health Education, University of Texas at Austin, Austin, Texas, USA
[3] Graduate Institute of Sports Science, Taipei Physical Education College, Taipei, Taiwan
[4] Graduate Institute of Sports Science, National Taiwan Sport University, Taoyuan, Taiwan

Reprint request to:
Mei-Chich Hsu
Graduate Institute of Sports Science
National Taiwan Sport University
Taoyuan, Taiwan
E-mail: meichich@gmail.com

ABSTRACT: In the present study we investigated the interactive effects of insulin and carbohydrate on glycogen replenishment in different rat hindlimb muscles. Forty male Sprague Dawley rats were assigned to 5 groups, including 1) sedentary control with carbohydrate supplement (2 g glucose · kg body wt^{-1}), 2) sedentary rats with 16 hours recovery, carbohydrate and insulin (0.5 U · kg body wt^{-1}), 3) swimming without recovery, 4) swimming with 16 hours recovery and carbohydrate supplement, and 5) swimming with 16 hours recovery, carbohydrate and insulin. The swimming protocol consisted of two 3 h swimming sections, which were separated by a 45 min rest. The insulin and carbohydrate were administered to the rats immediately after exercise. At the end of the experiment, the soleus (S), plantaris (P), quadriceps (Q) and gastrocnemius (G) were surgically excised to evaluate glycogen utilization and replenishment. We observed that glycogen utilization was significantly lower in G and Q than S and P during swimming (p <0.05), and S showed the greatest capacity of glycogen resynthesis after post-exercise recovery (p <0.05). In the sedentary state, the glycogen synthesis did not differ among hindlimb muscles during insulin and carbohydrate treatments. Interestingly, with insulin and carbohydrate, the glycogen resynthesis in S and P were significantly greater than in Q and G following post-exercise recovery (p <0.05). We therefore concluded that the soleus and plantaris are the primary working muscles during swimming, and the greatest glycogen replenishment capacity of the soleus during post-exercise recovery is likely due to its highest insulin sensitivity.

KEY WORDS: swimming exercise, soleus, plantaris, recovery

INTRODUCTION

Skeletal muscle is the primary depot of glycogen storage in the body. Additionally, skeletal muscle consists of diverse types of muscle fibres, which express varied contractile and metabolic properties (e.g. power output, glycogen store, etc.) [5,6]. In this regard, the storage and replenishment of muscle glycogen is critical for exercise capacity. Many previous investigations have thus focused on the impacts of exercise and insulin on glycogen metabolism in skeletal muscle [14,15]. Because there are various fibre-type compositions in different muscles, exercise results in diverse responses of glycogen utilization and resynthesis among different muscles. Moreover, the duration and intensity of exercise also lead to varied glycogen utilization and post-exercise glycogen synthetic capacity among different working muscles.

Optimizing muscle glycogen resynthesis is essential for subsequent exercise performance. It is well known that exercise-induced glycogen depletion can be rapidly replenished by enhancing glucose transport capacity during post-exercise recovery and that insulin can stimulate muscle glycogen synthesis through enhancing glucose transport and glycogen synthase activity [2,10]. Moreover, the combination of exercise, insulin and/or carbohydrate supplementation can produce additive effects on maximizing muscle glucose transport and glycogen synthesis [3]. However, the impacts of insulin and carbohydrate supplementation on exercise-induced glycogen utilization and post-exercise replenishment among different rat hindlimb muscles have not been well investigated.

Swimming exercise has been for years a widely used animal exercise model for investigating both acute and chronic physiological responses to exercise challenge [13,15,16,21]. However, most of the existing studies only compared different responses to exercise between different muscle fibre types within the same muscle (e.g. red and white portions) rather than investigating the diverse responses among muscle groups [13,14]. Since swimming is often used

for studies on exercise physiology and biochemistry in rodents, the understanding of muscle glycogen utilization and synthetic patterns in response to exercise among different muscle groups is practically important and is required for developing advanced sports nutrition strategies. Therefore, the purpose of the present study was to investigate the effect of insulin plus carbohydrate supplementation on glycogen replenishment among different hindlimb muscles in rats following prolonged swimming.

MATERIALS AND METHODS

Animal care: Forty male Sprague-Dawley rats (SD rats) at 7 weeks of age (weight: 200 g) purchased from the National Animal Laboratory of the NSC (National Science Council, Taipei, Taiwan, ROC) were housed in a cage of three and were provided food (rat chow, PMI Nutrition International, Brentwood Mo., USA) and water *ad libitum*. The temperature of our animal room was maintained at 23°C with a 12:12 h light-dark cycle. After arrival at our animal room, the rats were acclimated in our animal room for one week before the experiment.

Experimental design
1) sedentary rats with carbohydrate supplement (2 g glucose · kg body wt^{-1}), and 16 hours recovery (C), 2) sedentary rats with carbohydrate supplement, insulin administration (0.5 U · kg body wt^{-1}) and 16 hours recovery (I), 3) swimming exercise without recovery group (E0), 4) swimming exercise with carbohydrate supplement and 16 hours recovery (E16), and 5) swimming exercise with carbohydrate supplement, insulin administration and 16 hours recovery (IE16). In the sedentary groups (i.e. groups C and I), rats were given either carbohydrate or carbohydrate supplementation with insulin and then recovered in their cages for 16 h. In the acute

exercise group (E0), the rats were immediately anaesthetized following swimming exercise for muscle tissue harvesting. In the post-exercise recovery groups (i.e. E16 and IE16 groups), the rats were immediately given either carbohydrate or carbohydrate supplementation with insulin following swimming exercise and then recovered in their cages for 16 h. After completion of either exercise or recovery, the rats were then anaesthetized in preparation for muscle sampling. The experimental protocol is shown in Figure 1.

Swimming exercise protocol and recovery
The swimming exercise in the present study was performed as previously described [4,14]. In brief, after 12 h fasting, the swimming exercise protocol consisted of two sessions of 3 hours continuous swimming, and the two swimming sessions were separated by a 45 min rest. For the swimming protocol, three rats swam in a swimming cage, and the water temperature was continuously monitored and maintained at 34±1°C. Upon completion of the swimming exercise, the rats were brought back to their cage. For post-exercise recovery, the recovery period was 16 hours, and food and water were provided *ad libitum*. After completion of either exercise or recovery, all rats were anaesthetized by pentobarbital sodium (65.0 mg · kg body wt^{-1}) through an intraperitoneal injection. For muscle sampling, the middle belly part of the soleus (S), plantaris (P), quadriceps (Q), and gastrocnemius (G) from rat hindlimb was surgically excised and clamped frozen with tongs cooled in liquid nitrogen. These muscles were used for evaluating muscular glycogen utilization and replenishment. After muscle sampling, all rats were euthanized by cardiac injection of pentobarbital sodium (50.0 mg · kg body wt^{-1}).

Carbohydrate supplementation and insulin administration
The carbohydrate supplement was administered by oral gavaging. Rats from carbohydrate treatment groups (i.e. I, E16, and IE16)
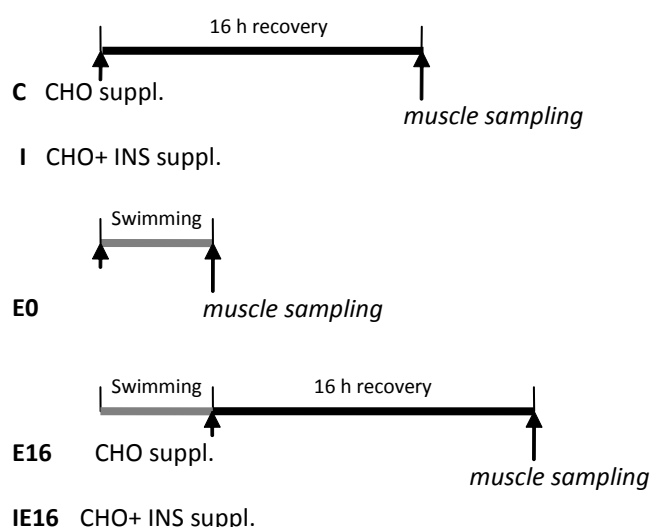

FIG. 1. EXPERIMENTAL PROTOCOL. CHO, CARBOHYDRATE; INS, INSULIN; SUPPL., SUPPLEMENTATION; C, SEDENTARY RATS WITH CHO SUPPLEMENT (2 g/kg BODY WT), AND 16 HOURS RECOVERY;; I, SEDENTARY RATS WITH CHO SUPPLEMENT, INS ADMINISTRATION (0.5 U/kg BODY WT) AND 16 HOURS RECOVERY; E0, SWIMMING EXERCISE WITHOUT RECOVERY GROUP; E16, SWIMMING EXERCISE WITH CARBOHYDRATE SUPPLEMENT AND 16 HOUR RECOVERY; IE16, SWIMMING EXERCISE WITH CARBOHYDRATE SUPPLEMENT, INSULIN ADMINISTRATION AND 16 HOURS RECOVERY

received 2 g·kg body wt of glucose[-1]. After the second session of swimming exercise, a bolus of carbohydrate supplement (50% w/v glucose water solution) was immediately given to all the rats, except for those in the C and E0 groups. Rat chow was continuously provided *ad libitum* during the post-exercise recovery period. For insulin administration, the rats in groups I and IE16 were injected with 0.5 U·kg body wt of insulin[-1] (Eli Lilly and Company, Indianapolis, Indiana, USA) intraperitoneally. Insulin was immediately administered following the carbohydrate supplementation.

Measurement of muscle glycogen
Muscle glycogen was determined by amyloglucosidase and glucose Trinder reactions. Frozen muscle samples were digested in 500 μl of 1 N KOH. Dissolved homogenates were neutralized by 50% glacial acetic acid and incubated in acetate buffer (0.3 M sodium acetate, pH to 4.8) containing amyloglucosidase (10 mg·ml^{-1}, Boehringer Mannheim, Mannheim, Germany) at room temperature. The reaction mixture was neutralized with 1 N NaOH. Free glucose was then analysed by measuring glucosyl units through the Trinder reaction (Trinder Glucose Kit, Sigma Chemical, St. Louis, MO).

Data calculation and statistics
The percentage of swimming-induced muscle glycogen depletion was calculated by the following formula: (C-E0)/C. In turn, the percentage of muscle glycogen replenishment during post-exercise recovery was calculated by the following formula: (E16-E0/E0). The increasing percentage of muscle glycogen in response to insulin was calculated by the following formula: (I-C/C). Finally, the percentage of muscle glycogen replenishment with insulin and carbohydrate treatment following post-exercise recovery was calculated by the following formula: (IE16-E0/E0). Abbreviations in calculations: C (basal muscle glycogen concentration), I (muscle glycogen concentration following 16 h recovery with insulin and carbohydrate treatment), E0 (post-exercise muscle glycogen concentration), E16 (muscle glycogen concentration following 16 h post-exercise reco-

very), and IE16 (muscle glycogen concentration following 16 h post-exercise recovery with insulin and carbohydrate treatment).

All the data were analysed using a one-way analysis of variance (one-way ANOVA). Significant differences among means were determined using Tukey post hoc analysis. Statistical differences were considered significant at the level $p<.05$, and all values were expressed as means ± S.E.

RESULTS
The basal muscle glycogen concentrations among different hindlimb muscles are shown in Figure 2. The basal glycogen content in gastrocnemius was significantly higher than in quadriceps, plantaris, and soleus (55.1 ± 2.05 vs. 36.0 ± 2.15, 26.4 ± 1.3 & 22.0 ± 1.07 $\mu mol·g^{-1}$, $p<0.05$). In addition, the quadriceps glycogen level was significantly higher than that of plantaris and soleus ($p<0.05$).

Figure 3 displays the depletion of glycogen following prolonged swimming in different hindlimb muscles. After prolonged swimming, gastrocnemius and quadriceps expressed a significantly lower percentage of muscle glycogen depletion than soleus and plantaris (53.2 ± 2.77 & 40.7 ± 4.46 vs. 75.3 ± 2.89 & $63.3\pm4.16\%$, $p<0.05$).
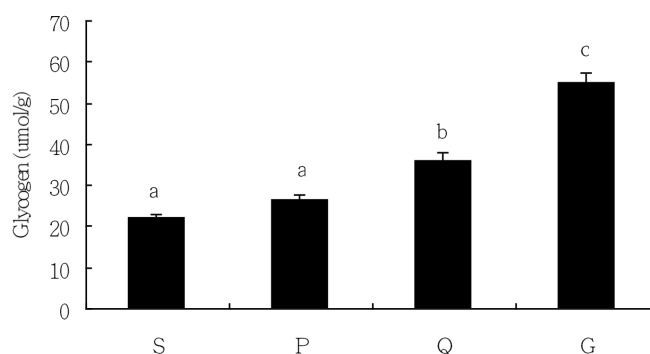

FIG. 2. BASAL GLYCOGEN CONCENTRATIONS IN DIFFERENT HINDLIMB MUSCLES.
Note: S, soleus; P, plantaris; Q, quadriceps;G, gastrocnemius. Values are expressed as mean ±S.E. in $\mu mol·g$ muscle wt[-1]. Mean values within a column with different superscript letters were significantly different ($p<0.05$)
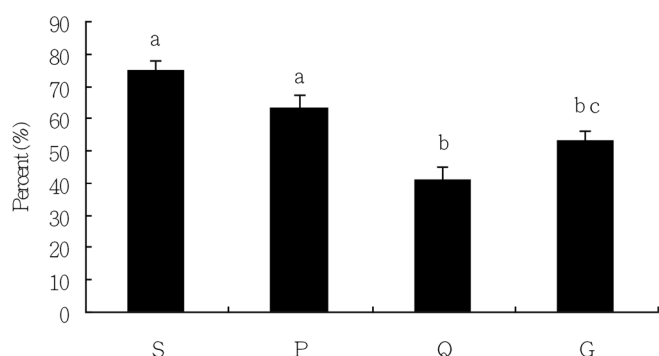

FIG. 3. THE PERCENTAGE OF GLYCOGEN DEPLETION FOLLOWING PROLONGED SWIMMING IN DIFFERENT HINDLIMB MUSCLES.
Note: S, soleus; P, plantaris; Q, quadriceps; G, gastrocnemius. Values are expressed as mean ±S.E. in percentage. Mean values within a column with different superscript letters were significantly different ($p<0.05$)

FIG. 4. THE PERCENTAGE OF POSTEXERCISE GLYCOGEN INCREMENT AFTER A 16 H RECOVERY IN DIFFERENT HINDLIMB MUSCLES.
Note: S, soleus; P, plantaris; Q, quadriceps; G, gastrocnemius. Values are expressed as mean ±S.E. in percentage. Mean values within a column with different superscript letters were significantly different ($p<0.05$)
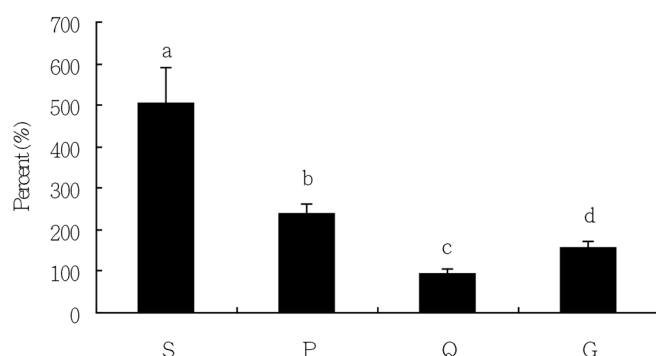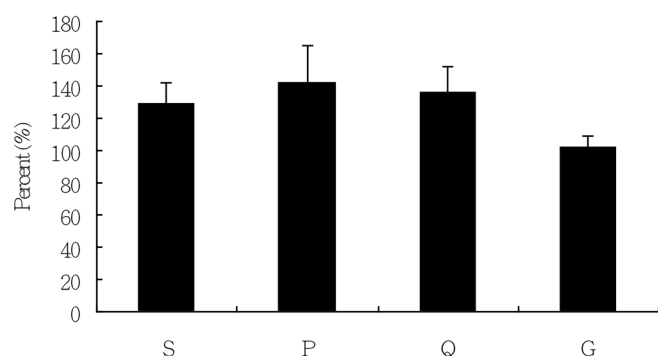
FIG. 5. THE PERCENTAGE OF GLYCOGEN INCREMENT AFTER A 16 HOURS WITH INSULIN INJECTION IN SEDENTARY RATS. S, SOLEUS; P, PLANTARIS; Q, QUADRICEPS; G, GASTROCNEMIUS. VALUES ARE EXPRESSED AS MEAN ± S.E. IN PERCENTAGE
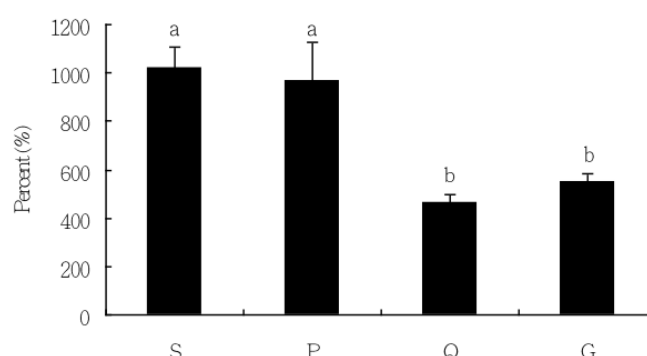

FIG. 6. THE PERCENTAGE OF POSTEXERCISE GLYCOGEN INCREMENT AFTER A 16 H RECOVERY WITH INSULIN AND CARBOHYDRATE SUPPLEMENTATION IN DIFFERENT HINDLIMB MUSCLES. S, SOLEUS; P, PLANTARIS; Q, QUADRICEPS; G, GASTROCNEMIUS. VALUES ARE EXPRESSED AS MEAN ± S.E. IN PERCENTAGE. MEAN VALUES WITHIN A COLUMN WITH DIFFERENT SUPERSCRIPT LETTERS WERE SIGNIFICANTLY DIFFERENT (P<0.05)

However, there were no differences in the percentages of muscle glycogen depletion between gastrocnemius and quadriceps. Additionally, the percentage of muscle glycogen depletion in soleus did not differ from that of plantaris.

The replenishments of glycogen following post-exercise recovery in hindlimb muscles are shown in Figure 4. After prolonged swimming, the percentage of glycogen replenishment following post-exercise recovery in soleus was significantly greater than in plantaris, quadriceps, and gastrocnemius (505.8 ± 85.3 vs. 238.7 ± 22.2, 92.6 ± 11.3, & $157.1 \pm 15.5\%$, p<0.05). However, there were no differences in the percentages of glycogen replenishment among plantaris, quadriceps, and gastrocnemius.

Figure 5 displays the insulin-induced increments of glycogen levels in hindlimb muscles. In the sedentary state, with insulin administration and carbohydrate supplementation, the increments of glycogen levels did not differ among the hindlimb muscles.

Figure 6 displays the replenishments of glycogen following post-exercise recovery with insulin in hindlimb muscles. After prolonged swimming, with insulin and carbohydrate supplementation, the percentages of muscle glycogen replenishment following post-exercise recovery in soleus and plantaris were significantly higher than in quadriceps and gastrocnemius (1023.5 ± 85.3 & 967.4 ± 161.4 vs. 463.6 ± 34.5 & $548 \pm 36.8\%$, p<0.05). However, there were no differences in the percentages of glycogen replenishment between gastrocnemius and quadriceps. Additionally, the percentage of glycogen replenishment in soleus did not differ from that in plantaris.

DISCUSSION

It has been documented that exercise-induced glycogen utilization and post-exercise glycogen replenishment in skeletal muscle are expressed in fibre-type specific manners. Swimming is a widely used experimental modality for investigating the physiological responses to exercise challenge, whereas the existing reports focusing on the impact of swimming exercise on glycogen metabolism in different muscle groups are very limited. In this regard, we investigated the patterns of glyco-

gen utilization and resynthesis during and after swimming exercise in rat skeletal muscle. The primary findings of this study are 1) a greater rate of glycogen utilization was observed in soleus and plantaris during prolonged swimming exercise than in other hindlimb muscles, but soleus exhibited the highest rate of glycogen resynthesis after 16 h post-exercise recovery; 2) soleus and plantaris also showed higher rates of post-exercise glycogen resynthesis in response to insulin and carbohydrate supplementation compared to other hindlimb muscles.

To the best of our knowledge, there were no studies focusing on glycogen utilization among different hindlimb muscle groups during swimming exercise. Here we observed that soleus and plantaris muscles (S: 75% and P: 63% glycogen depletion) exhibited greater glycogen utilization during swimming exercise compared to quadriceps and gastrocnemius muscles (Q: 40% and G: 53% glycogen depletion) (Figure 3), suggesting that deep layers of calf muscles are primarily working muscles responsible for continuous ankle plantar flexions and stroking movements rather than the superficial calf and thigh muscles. Soleus predominately consists of type I fibres (84%), but plantaris predominately contains type II fibres (approximately 94%) [7,18]. The morphological and metabolic properties of soleus are completely distinct from plantaris, and this therefore raised a question why the swimming protocol in this study resulted in a similar rate of intramuscular glycogen depletion in these two muscles. The results can be explained by the findings of Yoshimura et al. [22]. They found that depletion of muscle glycogen occurs first in the slow (0-0.5 h), then the intermediate (2-4 h), and, finally, the fast intrafusal fibres (4-8 h) during prolonged swimming [22]. Thus, in this study, the 6 h prolonged swimming exercise might fully recruit both slow and fast-twitch fibres, thereby leading to similar glycogen depletion in soleus and plantaris muscles.

Muscle glycogen resynthesis following prolonged exercise is strongly associated with the capacity of glucose uptake, which is directly correlated with intracellular contents of GLUT4 protein [12,13]. In this study, soleus showed the greatest muscle glycogen replenishment among hindlimb muscles following 16 h post-exercise reco-

very (Figure 4). Compared to other hindlimb muscle groups, soleus consists of a higher portion of type I fibre, which expresses greater GLUT4 protein and insulin sensitivity [20]. Therefore, the relatively greater glycogen resynthesis in soleus following prolonged swimming might be due to the higher intracellular concentration of GLUT4 protein in this muscle.

It has been noted that the muscular glycogen store is inversely associated with GLUT4 translocation and glucose transport activity [8,11]. In this study, there was no significant difference in glycogen depletion between soleus and plantaris, but we observed that our prolonged swimming protocol induced the greatest glycogen depletion in soleus (Figure 3). Thus, another possible explanation for greater glycogen replenishment in soleus was that more exercise-induced glycogen depletion might lead to higher glucose transport activity and greater glycogen resynthesis in soleus than in plantaris.

Insulin action varies among different muscles due to the differences in biochemical and metabolic properties [20]. Song et al. [20] used isolated muscle preparation to determine the muscle fibre type-specific response of intracellular signalling proteins to insulin. They reported that soleus muscle has the highest capacity of insulin-induced insulin receptor (IR) tyrosine phosphorylation and greater total protein expressions of phosphatidylinositol (PI)-3-kinase and protein kinase B (PKB/Akt), suggesting that the diversity of expression and activity of insulin signalling proteins contribute to fibre type-specific differences in insulin action in skeletal muscle. Here we observed that insulin treatment plus carbohydrate supplementation leads to significantly increased glycogen levels in all hindlimb muscles (Figure 5), but there were no differences in the increase in muscle glycogen among these muscles. These dissimilarities to our results might be due to the different experimental approaches. In this study, insulin administration plus glucose supplementation was followed by 16 h recovery, while Song et al. [20] exposed the isolated muscles in the presence of maximal insulin for only 40 min. It therefore suggested that the fibre type-specific differences in insulin-induced muscle glycogen synthesis might have gradually diminished during the period of 16 h post-exercise recovery.

Compared with several previous studies [17,19], our results showed that the diverse levels of muscle glycogen replenishment after post-exercise recovery are possibly associated with the exercise-specific muscle recruitment patterns in the presence of insulin and carbohydrate supplementation. Moreover, intensity and duration are the primary regulatory factors in muscle fibre recruitment during exercise. Armstrong et al. [1] previously reported that low exercise intensities primarily rely on type I fibres for contractile activity and that a major use of type II fibres only occurs during high intensity exercise or when the glycogen of type I fibres is depleted during prolonged low-intensity exercise [1]. Although all hindlimb muscles showed significant increase in glycogen resynthesis after post-exercise recovery, the glycogen resynthesis was much greater in soleus and plantaris (Figure 6). Together with previous and our present findings, we therefore speculated that swimming exercise primarily recruited soleus and plantaris, resulting in more glycogen depletion, thereby leading to greater insulin-stimulated glycogen replenishment in these two muscles. However, the fibre-type specific differences in glycogen synthesis between soleus and plantaris might disappear at the end of a 16 h post-exercise recovery period.

The activation of insulin signalling cascades is essential for muscle glycogen replenishment during the post-exercise recovery period. Both glucose transport and glycogen synthase activity are considered the key regulatory factors for glycogen biosynthesis in skeletal muscle, and the two major factors are highly regulated by insulin and exercise [9,14,15]. Additionally, the insulin action in skeletal muscle is expressed in a fibre type-specific manner, in part due to the diversity of expression and activity of insulin signalling proteins [20]. Here we found that, with insulin and carbohydrate supplementation, the post-exercise muscle glycogen replenishments were approximately 2-3-fold higher than swimming alone (Figures 4 and 6). However, this insulin-induced improvement in glycogen resynthesis was much higher in soleus and plantaris than in the other hindlimb muscle groups. Taken together, our results indicated that soleus and plantaris were primarily recruited during swimming, which reflected the greater synergistic effects of exercise and insulin on enhancing glycogen replenishment in these two muscles.

CONCLUSIONS

In summary, we found that soleus and plantaris exhibit greater rates of muscle glycogen depletion as compared to quadriceps and gastrocnemius, indicating that these two muscles are the primary working muscles in prolonged swimming exercise. Without insulin and carbohydrate supplementation, soleus and plantaris show higher capacity of glycogen replenishment after post-exercise recovery compared to other hindlimb muscles. Moreover, in both sedentary and swimming groups, insulin significantly increases muscle glycogen resynthesis in all hindlimb muscles, but the insulin-stimulated glycogen replenishment after post-exercise recovery is greater in soleus and plantaris. Our results therefore suggest that for future investigations using the swimming exercise model for determining muscle glycogen metabolism, soleus can be selected to represent type I fibre and plantaris to represent type II fibre.

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