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ORIGINAL ARTICLE

Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats

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Abstract Recent studies showed that a combination of carbohydrate and protein was more effective than carbohydrate alone for replenishing muscle glycogen after exercise. However, it remains to be unclear whether the source or degree of hydrolysis of dietary protein influences post-exercise glycogen accumulation. The aim of this study was to compare the effect of dietary protein type on glycogen levels in the post-exercise phase, and to investigate the effects of post-exercise carbohydrate and protein supplementation on phosphorylated enzymes of Akt/PKB and atypical PKCs. Male Sprague-Dawley rats, trained for 3 days, swam with a 2% load of body weight for 4 h to deplete skeletal muscle glycogen. Immediately after the glycogen-depleting exercise, one group was killed, whereas the other groups were given either glucose or glucose plus protein (whey protein, whey protein hydrolysates (WPH), casein hydrolysates or branched-chain amino acid (BCAA) solutions. After 2 h, the rats were killed, and the triceps muscles quickly excised. WPH caused significant increases in skeletal muscle glycogen level (5.01 ± 0.24 mg/g), compared with whey protein (4.23 ± 0.24 mg/g), BCAA (3.92 ± 0.18 mg/g) or casein hydrolysates (2.73 ± 0.22 mg/g). Post-exercise ingestion of glucose plus WPH significantly increased both phosphorylated Akt/PKB (131%) and phosphorylated PKCζ (154%) levels compared with glucose only. There was a significant positive correlation between skeletal muscle glycogen content and phosphorylated Akt/PKB ($r = 0.674$, $P < 0.001$) and PKCζ ($r = 0.481$, $P = 0.017$). Post-exercise supplementation with carbohydrate and WPH increases skeletal muscle glycogen recovery by activating key enzymes such as Akt/PKB and atypical PKCs.

Keywords Whey protein hydrolysates · Skeletal muscle glycogen · Exercise

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Introduction

Depletion of glycogen stores is known to be associated with fatigue during both sprint and endurance exercises (Hermansen et al. 1967; Karlsson and Saltin 1971) and, therefore, it is considered important to maintain adequate tissue stores of glycogen. Although it is well established that dietary carbohydrate is an effective source of tissue glycogen, studies have shown that a combination of carbohydrate and protein is more effective than carbohydrate alone in replenishing muscle glycogen in the 4-h period immediately after the exercise (Zawadzki et al. 1992; van Loon et al. 2000; Ivy et al. 2002). However, more studies have taken issue with the benefits of adding protein to carbohydrate supplement because the treatments were not isocaloric (Tarnopolsky et al. 1997; van Loon et al. 2000; Jentjens et al. 2001). Some studies showed that muscle glycogen resynthesis was similar when comparing a

carbohydrate plus protein and equal caloric carbohydrate supplement (Tarnopolsky et al. 1997; van Loon et al. 2000; Jentjens et al. 2001). In contrast, more recent studies demonstrated that the ingestion of carbohydrate plus protein produced greater glycogen resynthesis even when compared with isocaloric carbohydrate (Ivy et al. 2002; Berardi et al. 2006). However, there is only limited information on the effect of co-ingestion of carbohydrate with the different source (casein, whey protein) or degree of hydrolysis (non-hydrolyzed protein, protein hydrolysates and amino acids) of dietary protein on post-exercise glycogen repletion in skeletal muscle.

It is well known that certain dietary proteins affect skeletal muscle carbohydrate metabolism. There is evidence that the stimulation of plasma insulin is greater after the ingestion of whey protein compared with other protein sources such as cheese and milk (Nilsson et al. 2004). Differences in insulinotropic effect between various dietary proteins are likely to be associated with the increase in plasma amino acids concentration following ingestion; casein is digested slowly and induces a lower but more prolonged hyperaminoacidemia, whereas whey protein, which is digested quickly, induces higher postprandial retention of amino acids (Boirie et al. 1997). Furthermore, we have also shown that chronic feeding of whey protein increases glycogen content in liver (Morifuji et al. 2005b) and skeletal muscles in exercise-trained rats (Morifuji et al. 2005a) to a greater extent than casein. Thus, different digestive properties and amino acids composition of dietary protein may be associated with enhancing muscle glycogen synthesis.

It is well known that insulin stimulates glucose uptake in skeletal muscle tissue, mainly by initiating GLUT-4 translocation from intracellular pools to the plasma membrane, resulting in the stimulation of glycogen synthesis (Klip et al. 1993). Tyrosine phosphorylation of insulin receptor substrate-1 by insulin activates phosphoinositide 3-kinase (PI3-kinase) and induces activation of the downstream signaling molecules, protein kinase B (Akt/PKB) (Wang et al. 1999) and atypical protein kinase C (PKC)ζ and λ/ι (Bandyopadhyay et al. 1997). It is, therefore, important to examine phosphorylation of key enzymes, such as Akt/PKB and atypical PKC that regulate glucose uptake. Although it is well established that insulin is an effective means of maximizing glucose uptake in skeletal muscles, there is less information from nutritional studies on whether dietary components, such as protein, have beneficial effects on this process. Recently, leucine, isoleucine and branched-chain amino acids (BCAA)-containing dipeptides identified in whey protein hydrolysates (WPH), were shown to activate skeletal muscle glucose uptake via the PI3-kinase and atypical PKC pathways, a mechanism that is different from that involved in GLUT-4 translocation induced by insulin (Nishitani et al. 2002; Doi et al. 2003; Morifuji et al. 2009). However, the phosphorylation state of the enzymes that comprise these pathways has not been studied in vivo following the ingestion of carbohydrate and protein after exercise.

The aims of the present study were, therefore, to compare the effect of the source and degree of hydrolysis of dietary protein on glycogen levels in the post-exercise phase and to investigate the effects of post-exercise carbohydrate and protein supplementation on phosphorylated signaling molecules of Akt/PKB and atypical PKC, key enzymes regulating glucose uptake.

Materials and methods

Male Sprague-Dawley rats with body weights of approximately 150 g (CLEA Japan, Inc., Tokyo, Japan) were used in this study. All rats were housed individually in temperature-controlled rooms (22°C), with light from 8:00 a.m. to 8:00 p.m. and dark from 8:00 p.m. to 8:00 a.m. The study was approved by the animal committee of Food and Health R&D Laboratories, Meiji Seika Kaisha Ltd. with the animals receiving care under the guidelines laid down by this committee.

Exercise protocol

Exercise training and glycogen depletion exercise protocols were followed by the modified procedure of Sonou et al. (2008). On the first day to become accustomed to swimming training, the rats swam without a load for 4 h in two 2 h sessions, separated by 30 min of rest in a barrel filled with water maintained at 35°C to a depth of 50 cm, so that the average surface area available to each animal was 170 cm^2. On the second and third days, the rats swam without a load for 6 h in two 3 h bouts, separated by 45 min of rest. Ren et al. (1994) reported that this exercise training caused a twofold increase in skeletal muscle GLUT-4 contents and mRNA levels, and insulin- and contraction-stimulated glucose uptake. On the final day, the rats swam for 4 h with a load equivalent to 2% of body weight in order to deplete skeletal muscle glycogen levels. Our preliminary study showed that skeletal muscle glycogen level before glycogen depletion exercise was 12.2 $\pm$ 2.4 mg/g (data not shown).

Experimental protocol

The experimental protocol is shown in Fig. 1. The rats had free access to food (protein 23.6%, fat 5.3%, carbohydrate 54.4%, ash 6.1%, fiber 2.9%, moisture 7.7%, MF, Oriental Yeast Co., Ltd., Osaka, Japan) and drinks. One day before the glycogen depletion exercise, the rats were fed with 5 g of a restricted diet (MF, Oriental Yeast Co., Ltd., Osaka,

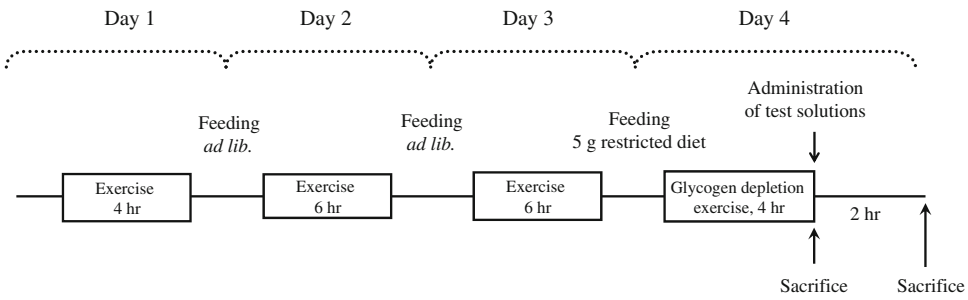

Fig. 1 The experimental protocol

Japan). Immediately following the glycogen depletion exercise, one group ($n = 8$ per group) was killed and the other groups ($n = 8$ per group) were given test solutions orally using a sonde [1.0 mL/100 g body weight (BW)]. Two hours after the ingestion of the test solutions, the remaining rats were killed under sodium pentobarbital anesthesia (40 mg/kg BW i.p.). Immediately after killing, the triceps muscles were excised quickly, washed and then frozen at −80°C until assay.

Preparation of test solution

Whey protein (Tatua Co-operative Dairy Company Limited, New Zealand), WPH (Meiji Seika Kaisha Ltd., Japan), BCAA (Ajinomoto Co., Inc., Japan) and casein hydrolysates (DSM Food Specialties, Netherland) were used. The protein content of these preparations was measured by the Kjeldahl method (Bradstreet 1954). The amino acid composition of the preparations is shown in Table 1. The average chain length of the peptides was calculated as the ratio of total nitrogen (TN) to amino nitrogen (AN) in the protein samples. Average peptide length, calculated as the TN/AN ratio, was similar in the whey protein (TN/AN = 3.64) and casein hydrolysates (TN/AN = 4.63).

Solutions of glucose [30% (w/v)], glucose plus whey protein, WPH, casein hydrolysates (30% glucose plus 10% protein) and glucose plus BCAA (30% glucose plus 1.16% leucine, 0.54% isoleucine, 0.54% valine) were prepared. The total amount of BCAA was similar to their content in whey protein (Table 1).

Table 1 Amino acid composition of test diets (%)

	Whey protein and whey protein hydrolysates	Casein hydrolysates
Ala	4.78	3.05
Arg	2.95	3.46
Asx	10.52	6.37
Cys	2.55	0.13
Glx	16.81	21.08
Gly	1.92	1.79
His	2.19	2.99
Ile	5.40	4.57
Leu	11.57	9.47
Lys	9.44	7.31
Met	2.03	2.96
Phe	3.61	4.52
Pro	5.30	10.56
Ser	4.92	5.40
Thr	5.22	3.98
Trp	1.90	1.00
Tyr	3.49	5.02
Val	5.37	6.32
IAA	46.73	43.13
BCAA	22.34	20.36

Indispensable amino acids (IAA): Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, His. Branched-chain amino acids (BCAA): Val, Leu, Ile

Muscle glycogen contents

Perchloric acid extracts of muscle were assayed for glycogen by the amyloglucosidase method (Passonneau and Lauderdale 1974).

Western blotting

Muscle samples (50 mg) were weighed and homogenized in ice-cold buffer pH 7.4 containing 50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 1.0 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 2.0 mM Na_3VO_4, aprotinin 10 μg/ml, leupeptin 10 μg/ml, pepstatin 0.5 μg/ml and PMSF 0.5 μg/ml. The homogenates were centrifuged at 4,000*g* for 30 min at 4°C and the supernatants then aliquoted into several test tubes and stored at −80°C for later analysis. The total protein concentration of the tissue homogenate supernatants was measured using bicinchoninic acid with bovine serum albumin as the standard. Protein phosphorylation was determined using Western blotting. Equal amounts of muscle proteins (50 μg for phosphorylated Akt/PKB and phosphorylated PKCζ) were separated by gel electrophoresis, using sodium dodecyl sulfate (SDS)-PAGE, 5.0–10% gradient gels (Perfect NT Gel, DRC, Co., Ltd., Tokyo, Japan). The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Hybond-P,

Amersham Biosciences, Piscataway, NJ, USA) and blotted for 1 h at room temperature with gentle agitation in freshly prepared TBS containing 0.5% non-fat dry milk and 0.1% tris-buffered saline with tween-20. The PVDF membranes were incubated with gentle agitation at 4°C over night with a primary antibody, which for phosphorylated Akt/PKB was rabbit anti-phospho-Akt (Ser^{473}) (1:1,000 dilution; Cell Signaling Technology, Beverly, MA, USA), and for phosphorylated PKCζ was rabbit anti-phospho-PKCζ (Thr^{410}) (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the membranes were washed in 0.1% Tris-buffered saline–tween 20 solution they were incubated with a secondary reagent for 1 h at room temperature with agitation. An anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Beverly, MA, USA) was used at a dilution of 1:3,000 for both phosphorylated Akt/PKB and phosphorylated PKCζ. The PVDF membranes were then washed in 0.1% Tris-buffered saline–tween 20, and the antibody-bound proteins visualized using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Amersham, UK) according to the manufacturer's protocol. The images were visualized using the Chemi-doc Gel Quantification System (Bio-Rad, Hercules, CA, USA).

Statistics

Data were expressed as mean ± SEM. The significance of differences between the means of the test and control groups was determined by one-way ANOVA and Tukey's post hoc analyses. Associations between the variables were examined using Pearson's correlation coefficients. Differences between groups were considered to be statistically significant at $P < 0.05$.

Results

Skeletal muscle glycogen contents

Skeletal muscle glycogen content post-exercise and after 2 h of recovery are shown in Fig. 2. The addition of the proteins to the glucose solution, with the exception of casein hydrolysates, resulted in significant increases in muscle glycogen content, compared with glucose only. In particular, WPH caused greater increases in post-exercise skeletal muscle glycogen content than whey protein, BCAA and casein hydrolysates.

Skeletal muscle phosphorylated Akt/PKB and PKCζ levels

To determine the effect of co-ingestion of glucose and dietary protein on phosphorylated Akt/PKB and PKCζ, the key enzymes that regulate glucose uptake, we selected the groups of glucose and glucose plus whey hydrolysates that caused maximum post-exercise stimulation of skeletal muscle glycogen repletion. Post-exercise ingestion of glucose significantly increased phosphorylated Akt/PKB levels, compare to the immediate post-exercise levels, whereas phosphorylated PKCζ levels did not change. Ingestion of glucose plus WPH after exercise significantly increased both the phosphorylated Akt/PKB and PKCζ levels, compared with the immediate post-exercise and ingestion of glucose only (Fig. 3).

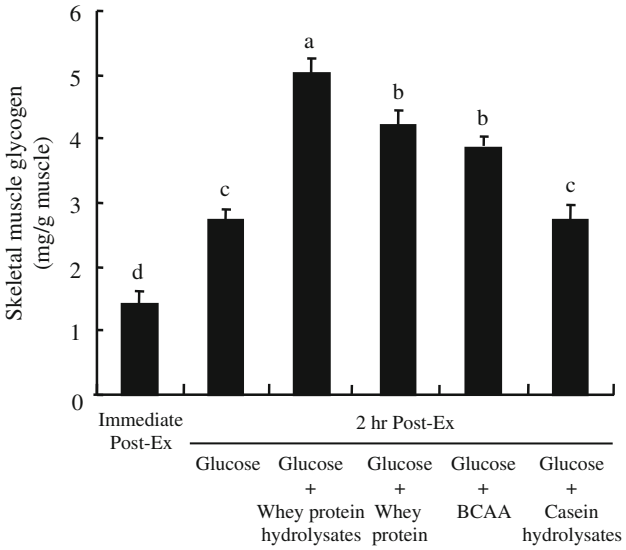

Fig. 2 Skeletal muscle glycogen content immediately after exercise and after 120 min of the recovery period, grouped according to the different type of test drinks ingested ($n = 8$ per group). Values are mean ± SEM. Mean values within a column with unlike *superscript letters* were significantly different ($P < 0.05$)

Correlation between glycogen content and phosphorylated Akt/PKB and PKCζ in skeletal muscle

We showed there was a significant positive correlation between skeletal muscle glycogen content and levels of phosphorylated Akt/PKB and phosphorylated PKCζ (Fig. 4).

Discussion

Several studies have reported that the co-ingestion of protein with lower quantities of carbohydrate (≤0.8 g/kg h^{-1}) accelerate post-exercise muscle glycogen synthesis when compared with the ingestion of carbohydrate only in human (Zawadzki et al. 1992; Tarnopolsky et al. 1997; van Loon et al. 2000; Ivy et al. 2002). However, it is not clear whether the source and degree of hydrolysis of dietary

protein ingested with carbohydrate influences post-exercise glycogen accumulation in skeletal muscle. This study showed that the ingestion of carbohydrate plus WPH was more effective for increasing post-exercise skeletal muscle glycogen content in rats than ingestion of other protein sources.

We found that the source and hydrolysis of dietary protein affected post-exercise glycogen repletion in skeletal muscles. WPH caused significantly greater increases in glycogen contents than either whey protein, casein hydrolysates or BCAA. Possible explanations for the increase in skeletal muscle glycogen levels are the amino acid composition and degree of hydrolysis of dietary proteins. A recent study demonstrated that amino acids, in particular BCAA, stimulated glycogen synthesis in skeletal muscle. In that study, Armstrong et al. (2001) used cultured human muscle cells to show that amino acids stimulated p70S6 kinase and caused transient inhibition of glycogen synthase kinase-3 (GSK-3), thereby increasing glycogen synthesis. Peyrollier et al. (2000) also showed leucine-stimulated glycogen synthesis in L6 cells as a result of inactivating GSK-3, while Doi et al. (2005) reported that leucine caused a significant increase in glucose incorporation into intracellular glycogen in vivo studies. In addition, the present study showed that skeletal muscle glycogen content following co-ingestion of glucose and BCAA reached the same level as that attained with glucose and whey protein. Taken together, these results indicate that the BCAA content, especially leucine, in whey protein plays an important role in post-exercise glycogen recovery.

However, comparison of the different types of protein showed that despite the BCAA levels in whey protein and casein hydrolysates being similar, skeletal muscle glycogen contents were different following the supplementation of the two hydrolysates. Furthermore, ingestion of WPH caused significantly greater increases in skeletal muscle glycogen levels than non-hydrolyzed whey protein even though their amino acid composition was the same. These results, therefore, indicated that another factor, other than the amino acid composition of dietary protein, contributed to post-exercise glycogen repletion. Recently, we demonstrated in an in vitro study that BCAA-containing bioactive peptides, such as Ile–Val, Leu–Val, Val–Leu, Ile–Ile, Ile–Leu, Leu–Ile, and Leu–Leu increased the rate of glucose uptake, resulting in the accumulation of glycogen (Morifuji et al. 2009). Whey protein and casein hydrolysates contain 21.63 and 3.31 mg/g BCAA-containing bioactive peptides, respectively. Therefore, certain bioactive peptides, such as BCAA-containing peptides, may also contribute to enhanced post-exercise skeletal muscle glycogen levels.

Although it is well known that the ingestion of protein with carbohydrate has a beneficial role in the rate of muscle glycogen storage after exercise, compared with carbohydrate alone, there is only limited in vivo data demonstrating how downstream signaling molecules are changed after the ingestion of carbohydrate and protein drinks. This study showed that post-exercise ingestion of glucose plus WPH in rats increased both Akt/PKB and PKCζ phosphorylation, compared with glucose alone.

It is possible that skeletal muscle Akt/PKB and PKCζ may be activated by both insulin-dependent- and insulin-independent mechanisms. Insulin is a strong activator of muscle glycogen synthesis owing to its stimulating effect on glucose transport via activation of Akt/PKB, atypical PKC and glycogen synthase. Although pancreatic insulin secretion is regulated primarily by the concentration of blood glucose, protein as well as some amino acids also stimulates insulin secretion. In situations when protein and carbohydrate are consumed together, the insulin response is greater than that predicted by the sum of the individual responses. Furthermore, milk proteins have insulinotropic properties, with the whey fraction being a more efficient insulin secretagogue than casein (Gannon et al. 1992; Nilsson et al. 2004; Tessari et al. 2007). As a consequence, co-ingestion of glucose and WPH stimulate insulin release to a greater extent than glucose alone suggesting that this elevation in insulin level may in turn activate Akt/PKB and PKCζ in skeletal muscle.

A recent study demonstrated that certain amino acids directly activate the key proteins in the insulin signaling pathway. In that study, Nishitani et al. (2002) confirmed that increased glucose uptake induced by leucine was inhibited completely by pretreatment with LY294002, a PI-3 kinase inhibitor, and GF109203X, an atypical PKC inhibitor. Other studies also showed leucine failed to stimulate Akt/PKB, indicating that signaling pathways activated by insulin and growth factors may not be necessary or present at sufficiently high levels to mediate the effects of amino acids on glucose uptake (Peyrollier et al. 2000; Greiwe et al. 2001). This led the authors to conclude that the downstream signal was different from that of insulin-stimulated glucose uptake. Furthermore, we have reported previously that BCAA-containing bioactive peptides, identified in WPH, increased glucose uptake via the PI3 kinase and atypical PKC pathways in both L6 myotubes and isolated muscles (Morifuji et al. 2009). These findings, therefore, indicate that certain amino acids and peptides may act directly to activate PKCζ. Taken together these results suggest that the activation of Akt/PKB is related to insulin secretion, whereas the activation of atypical PKC involves both amino acids and insulin action.

In this study, we also confirmed a significant positive correlation between phosphorylated Akt/PKB, phosphorylated PKCζ and skeletal muscle glycogen content. However, it has become increasingly clear that both Akt/PKB

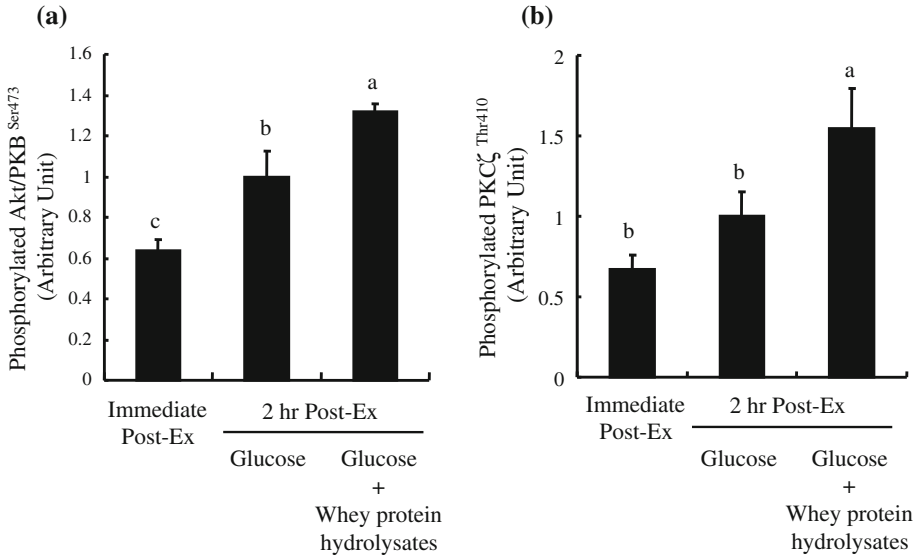

Fig. 3 Phosphorylated **a** Akt/PKB and **b** PKCζ immediately after exercise and after 120 min of the recovery period, with the ingestion of either glucose or glucose plus whey protein hydrolysates ($n = 8$ per group). Values are mean ± SEM. Mean values within a column with unlike *superscript letters* were significantly different ($P < 0.05$)

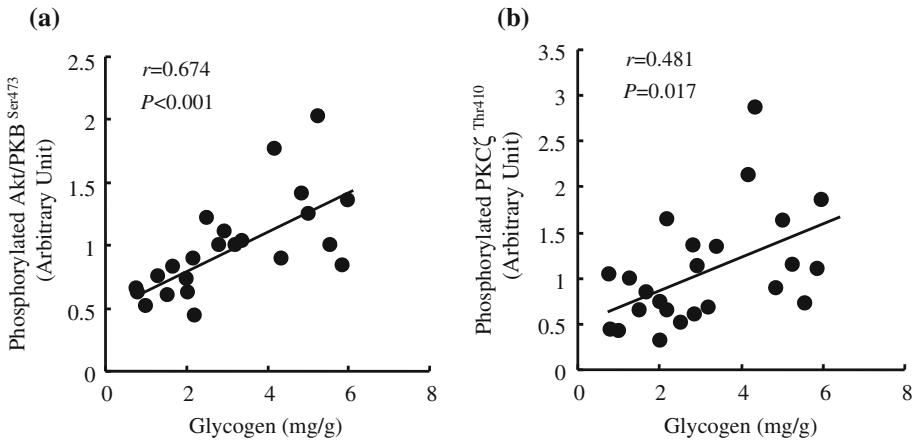

Fig. 4 Correlation between skeletal muscle glycogen contents and phosphorylated **a** Akt/PKB and **b** PKCζ

and atypical PKC are important for mediating the glucose transport effects of insulin, while Akt/PKB, rather than atypical PKC, appears to be important for stimulating glycogen synthesis and promoting glucose storage in muscle. Therefore, co-ingestion of glucose and WPH may regulate glucose uptake primarily by activating Akt/PKB and atypical PKC, resulting in the stimulation of glycogen synthesis by Akt/PKB.

In conclusion, our results demonstrate that the ingestion of carbohydrate plus WPH was more effective for increasing post-exercise skeletal muscle glycogen content in rats than ingestion of other protein sources. Post-exercise supplementation with carbohydrate and WPH may function to increase skeletal muscle glycogen recovery in order to activate Akt/PKB and atypical PKC, two key enzymes involved in the regulation of glucose uptake.

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