Effects of a Very High-Carbohydrate Diet and Endurance Exercise Training on Pancreatic Amylase Activity and Intestinal Glucose Transporter Content in Rats

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Summary
We previously reported that the combination of a very high-carbohydrate diet and endurance training increased glucose transporter 4 and glycogen concentration in skeletal muscle. However, it remains unclear whether they also affect the digestive and absorptive capacity in the pancreas and small intestine, which are suggested to be rate-limiting steps in the delivery of exogenous carbohydrates to skeletal muscle and muscle glycogen synthesis. Thus, we aimed to evaluate the effects of a very high-carbohydrate diet and endurance training on pancreatic amylase activity and intestinal glucose transporters in rats and to examine the relationship between these adaptations and their influence on muscle glycogen concentration. Male Sprague–Dawley rats (n=29) were fed a high-carbohydrate diet (59% carbohydrate) or a very high-carbohydrate diet (76% carbohydrate) for 4 wk. Half of the rats in each dietary group were subjected to 6-h swimming exercise training (two 3-h sessions separated by 45 min of rest) for 4 wk. Although there was no significant effect of diet or endurance training on sodium-dependent glucose transporter 1 and glucose transporter 2 contents in the intestine, the rats fed a very high-carbohydrate diet in combination with endurance training had substantially higher pancreatic amylase activity and muscle glycogen concentration. Furthermore, there was a positive correlation between pancreatic amylase activity and muscle glycogen concentration (r=0.599, p=0.001). In conclusion, intake of a very high-carbohydrate diet and endurance training synergistically elevated carbohydrate digestive capacity, which partially accounted for the higher muscle glycogen accumulation.

Key Words
very high-carbohydrate diet, exercise training, glycogen, amylase, SGLT1, GLUT2, skeletal muscle, pancreas, small intestine

Endogenous carbohydrate (CHO), which is stored as glycogen, is widely recognized as an important energy source for prolonged intense exercise. Fatigue during exercise is often associated with muscle glycogen depletion (1–3). Thus, it is necessary to increase muscle glycogen levels before exercise to maintain exercise intensity and performance and to delay fatigue in events such as a marathon (4, 5).

Kenyan athletes are famous for their outstanding performance in long-distance races in recent years. Interestingly, Onywera et al. (6) reported that they consume a very high-CHO, low-fat diet (70–80% CHO and <20% fat as total energies), whereas a relatively high-CHO diet (<60% CHO) has been recommended for endurance athletes in current sports nutrition guidelines (7). We recently investigated the effect of 4-wk feeding with a very high-CHO diet (76% of total energy), combined with endurance exercise training (6-h swimming exercise per day, 5 d per week) on glycogen metabolism in rat skeletal muscle (8). As a result, a very high-CHO diet feeding and endurance exercise training additively increased muscle glucose transporter 4 (GLUT4) content, which is a key regulatory molecule for the uptake of glucose in skeletal muscle (9) and thus elevates muscle glycogen concentrations (8). These results suggest that a very high-CHO diet-induced increase of GLUT4 and glycogen levels in skeletal muscle may account for the superior performance of Kenyan long-distance runners.

The gastrointestinal (GI) tract is the rate-limiting step in the delivery of endogenous CHO to skeletal muscle. We previously reported that long-term endurance exercise training resulted in not only higher muscle GLUT4 content, but also larger increase in the activity of amylase, which is the most important digestive enzyme for CHO, in pancreas (10). Furthermore, strong significant cor-
relation was observed between muscle glycogen concentration and pancreatic amylase activity, suggesting that the increase in digestive capacity as well as GLUT4 content may be involved in the elevated muscle glycogen by chronic exercise training (10). On the other hand, previous studies have shown that long-term intake of high-CHO diets can increase amylase content in the pancreas (11–14). These findings indicate that CHO digestive capacity may adapt to the diet as well. Therefore, it is likely that our previous findings that higher muscle glycogen levels resulted from feeding a very high-CHO diet (11–14). These findings indicate that CHO digestive capacity may also contribute to the elevation of muscle glycogen by long-term exercise training.

Digested CHOs are transported across the brush border membrane by sodium-dependent glucose transporter 1 (SGLT1), and they pass through the basolateral membrane into the circulation via glucose transporter 2 (GLUT2)-mediated facilitated diffusion (15). Because 90% of glucose absorption in the small intestine is mediated by these glucose transporters (16), their expression is likely to be a major determinant of intestinal glucose absorptive capacity. Our previous study has shown that long-term endurance training increased intestinal SGLT1 protein content, and this was associated with increased muscle glycogen concentration (10). These results suggest that the increase in CHO absorptive capacity may also be involved in the elevated muscle glycogen by long-term exercise training. In addition, it is known that intestinal SGLT1 content is increased by long-term intake of high-CHO diets as well (17, 18), whereas Moran et al. (17) reported that the increase in CHO content of diet from 52.6% to 60.3% did not increase SGLT1 content in the intestine. These findings suggest that intestinal SGLT1 content is already developed in rats fed the control high-CHO diet (59% of total energy) of our previous study, and no further increase may be observed in rats fed the very high-CHO diet (76% of total energy). Therefore, although CHO absorptive capacity may also contribute to the endurance exercise training- and very high-CHO diet-induced increases in the muscle glycogen levels observed in our previous study, the magnitudes of these relationships may be smaller than the association with muscle GLUT4 content or pancreatic amylase activity.

Against this background, the purpose of this study was to evaluate the effect of 4 wk of very high-CHO diet in combination with endurance exercise training on pancreatic amylase activity and intestinal glucose transporter content in rats by analyzing the samples of pancreas and small intestine that were taken from our previous study (8) and to examine the relationship between these adaptations and muscle glycogen concentration.

MATERIALS AND METHODS

The following methods have been reported in detail previously (8): animals, experimental diet, and training protocol and are summarized below for the convenience of the readers.

**Animals and protocol.** Four-week-old male Sprague–Dawley (SD) rats (CLEA Japan, Inc., Tokyo, Japan) were housed in individual cages. The environment was maintained at 22±1°C, with 50±5% humidity and lights turned on from 09:00 am to 21:00 pm. All animals were treated in accordance with the national guidelines for the care and use of laboratory animals (Notification of the Prime Minister’s Office of Japan). The Animal Experimental Committee of the University of Tokyo approved the experimental protocol (approval no. 29-10).

During an acclimatization period of approximately 1 wk, all rats were fed a high-CHO diet (19.2% protein, 21.6% fat, and 59.2% CHO as a percentage of total energy, 4.17 kcal/g), which was based on the AIN-93G formula (19). After this acclimatization period, the rats were divided into two dietary groups: one group that continued with a high-CHO diet (HCHO group, n=14) and another group that was fed a very high-CHO diet (VHCHO group, n=15: 21.5% protein, 2.4% fat, and 76.1% CHO as a percentage of total energy, 3.72 kcal/g). The composition of the very high-CHO diet increased the proportion of CHOs to the same level as the Kenyan runners’ diets, as reported in previous studies (6) and reduced the proportion of fat to the minimum amount that could prevent deficiency of essential fatty acids (20). The mean body weight and food efficiency during the acclimatization period were matched between the two groups. Rats in each group continued to consume these diets for 4 wk. All rats were allowed free access to the experimental diets and water, and their food intakes and body weights were recorded every other day.

Rats in each dietary group were further divided into either a sedentary (HCHO-Sed or VHCHO-Sed groups, n=7 each) or an exercise training (HCHO-Tr or VHCHO-Tr groups, n=7 or 8) group. Rats in the HCHO-Tr and VHCHO-Tr groups performed the swimming exercise for 6 h (two 3-h exercise sessions, separated by 45 min of rest) each day. This level of training was maintained for 4 wk (5 times per week). All animals were acclimated to swimming exercise for 10 min per day for 2 d before starting each exercise training. Rats in the exercise training groups swam in a barrel filled to a depth of 45 cm in groups of 7 or 8. The average surface area was 200–230 cm²/rat. The temperature of the water was maintained at ±1°C during the swimming exercise (10, 21).

At 18 h after the last training session, the rats were sacrificed under anesthesia with isoflurane. The epididymal fat tissue and pancreas were removed, weighted, and frozen in liquid N₂. The jejunum was dissected and washed in 0.9% NaCl, after which the mucosa was scraped with a spatula and stored in a Radio-Immuno Precipitation Assay (RIPA) lysis buffer (Merck Millipore, Billerica, MA) containing 50 mM Tris- HCl pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mM ethylenediaminetetraacetic acid (EDTA) with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), previously dispensed in a 1.5 mL microtube (10). The samples were stored at −80°C until analysis.

**Pancreatic amylase activity measurement.** A portion
of the pancreas was homogenized in ice-cold assay buffer and centrifuged at 12,000 rpm for 5 min at 4°C. Amylase activity in the supernatant was analyzed using the Amylase Assay kit (Abcam, Cambridge, UK) according to the manufacturer’s instructions.

Sample homogenization. Another portion of the pancreas and jejunal samples were homogenized in ice-cold RIPA lysis buffer with a protease inhibitor cocktail. Pancreatic protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA). The homogenate of the jejunal was subjected to 3 freeze-thaw cycles to disrupt intracellular organelles before being rotated end-over-end at 4°C for 60 min to solubilize the protein. The homogenized jejunal sample was centrifuged at 700 \times g for 5 min at 4°C, after which the supernatant was collected.

Western blot analysis. A BCA protein assay kit was used to measure the protein concentration in the supernatant of the jejunal. Sample was prepared in a Laemml buffer consisting of 277.8 mM Tris-HCl, 44.4% (w/v) glycerol, 4.4% (w/v) lithium dodecyl sulfate, and 0.02% (w/v) bromophenol blue, at pH 6.8 (Bio-Rad Laboratories, Hercules, CA) with dithiothreitol (Bio-Rad). The mixture was heated at 95°C for 5 min in a heating block. The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (% of polyacrylamide in resolving gels: 7.5% for SGLT1 and 10% for GLUT2) and then transferred to polyvinylidene difluoride membranes (Merck Millipore) at 200 mA for 90 min. Following transfer, the membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) with 0.1% Tween20 (TBS-T) supplemented with 5% (w/v) nonfat powdered milk. The membranes were incubated at 4°C overnight with a primary antibody dilution of 1:1,000 in TBS-T containing 5% bovine serum albumin using anti-GLUT2 (#07-1402; Merck Millipore) and anti-SGLT1 (#07-1417; Merck Millipore). The membranes were then incubated at room temperature for 1 h with secondary antibodies (goat anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:5,000 in TBS-T containing 1% nonfat powdered milk. The resulting bands were visualized using an enhanced chemiluminescence prime reagent (GE Healthcare, Buckinghamshire, UK) and quantified using Image Studio Digits (Ver. 5.2; LI-COR Biosciences, Lincoln, NE). To verify equal protein loading across lanes, the membranes were stained with Ponceau (Sigma-Aldrich).

Glycogen measurement. The epitrochlearis muscle was homogenized with 0.3 M perchloric acid. After acid hydrolysis, glycogen concentration was measured using enzymatic methods described by Lowry and Passonneau (23).

Statistical analysis. Data are presented as the mean± standard error of the mean (SE). Two-way analysis of variance (ANOVA) was performed to determine the effects of exercise training with two levels: with or without swimming exercise training, and of diet: HCHO or VHCHO diets. The Tukey–Kramer test for multiple comparisons was used for post-hoc analyses when a significant interaction was detected using ANOVA. Partial eta-squared (\(\eta^2\)) and Cohen’s d were calculated as measures of effect size for the ANOVAs and the post hoc analyses, respectively. In addition, statistical power was calculated using G*Power software (version 3.1). Associations between variables were determined using the Pearson’s correlation coefficients. All statistical analyses were performed using BellCurve for Excel software (Social Survey Research Information, Tokyo). A value of \(p<0.05\), was defined as the threshold of statistical significance.

RESULTS

Final body weight, total energy intake, and total CHO intake
The final body weight, total energy intake, and total CHO intake are shown in Table 1. We used data from our previous study on initial body weight, final body weight, and total energy intake (8). Although no significant interaction between exercise training and diet was observed on the final body weight, there was a main effect of exercise training. The final body weight of the Tr group was significantly lower than that of the Sed group (\(p<0.001\)). Furthermore, a significant main effect of diet on final body weight was observed, with the VHCHO group showing significantly lower final body weight compared with the HCHO group (\(p<0.001\)).

There was no significant interaction between exercise training and diet on total energy intake or total CHO intake. A significant main effect of exercise training on total energy intake and total CHO intake was observed, which was significantly lower in the Tr group than in the Sed group (\(p<0.001\)). In addition, there was also a main effect of diet on total CHO intake, with the VHCHO group having significantly higher total CHO intake compared with the HCHO group (\(p<0.001\)).

Pancreas weight and pancreatic protein concentration
Pancreas weight and pancreatic protein concentration are shown in Table 1. We found no significant interaction between exercise training and diet on these parameters. Conversely, the main effect of exercise training was on pancreatic protein concentration; the Tr group had significantly higher pancreatic protein concentration than the Sed group (\(p<0.001\)). Furthermore, a significant main effect of diet on pancreas weight and pancreatic protein concentration were observed, which were significantly higher in the VHCHO group than in the HCHO group (\(p=0.002\) and \(p=0.002\), respectively).

Pancreatic amylase activity
There was a significant interaction between exercise training and diet on pancreatic amylase activity (\(p<0.001\), partial \(\eta^2=0.38, 1-\beta=0.98\)). Pancreatic amylase activity in the VHCHO-Tr group was significantly higher compared with those in the HCHO-Sed, HCHO-Tr, and VHCHO-Sed groups (vs. HCHO-Sed \(p<0.001\), \(d=6.15, 1-\beta=1.00\); vs. HCHO-Tr \(p<0.001\), \(d=3.92, 1-\beta=1.00\); vs. VHCHO-Sed \(p<0.001\), \(d=...
training (p<0.001, partial \( \eta^2 = 0.13, 1 - \beta = 0.25 \)). Although no significant interaction between exercise training and diet was observed on glycogen concentration in the epitrochlearis muscle (p=0.08, partial \( \eta^2 = 0.07, 1 - \beta = 0.21 \)), there was a main effect of exercise training (p<0.001, partial \( \eta^2 = 0.17, 1 - \beta = 0.65 \)) on muscle glycogen concentration observed, with the VHCHO group showing significantly higher glycogen concentration in the epitrochlearis muscle compared with the HCHO group.

### DISCUSSION

The major findings of this study were that 4 wk of feeding high-CHO diet combined with endurance exercise training synergistically increased the pancreatic amylase activity in rats and that the enhanced pancreatic amylase activity was significantly associated with an increase in glycogen content in rat skeletal muscle.

Our previous study (10) and others (24–26) have reported that 6 to 8 wk of endurance exercise training itself substantially increased pancreatic amylase activ-
Contrary to these findings, this study showed that 4 wk of endurance exercise training with feeding of the high-CHO diet (HCHO group) did not induce any change in pancreatic amylase activity (Fig. 1). Since the sufficient adaptation of glucose metabolism function in skeletal muscle was confirmed at the end of 4-wk endurance exercise training in our previous study (8), we considered that the pancreatic amylase activity may also be increased by 4 wk of endurance exercise training. However, this result suggests that more than 4 wk of training are required to induce enzymatic adaptation in the pancreas. Therefore, future studies need to investigate in more detail the training period required to induce adaptation of pancreatic amylase activity.

Fig. 2. Effects of a very high-CHO diet and endurance exercise training on GLUT2 (A) and SGLT1 (B) content in the jejunum. Values are means±SE. n=7–8. Data were analyzed using two-way ANOVA. HCHO, high-CHO diet group; VHCHO, very high-CHO diet group; Sed, sedentary group; Tr, exercise training group.

Fig. 3. Effects of a very high-CHO diet and endurance exercise training on GLUT4 content (A) and glycogen concentration in the epitrochlearis muscle (B), and relationship between muscle glycogen concentration and muscle GLUT4 content (C) or pancreatic amylase activity (D). Values are means±SE. n=7–8. Data were analyzed using two-way ANOVA. HCHO, high-CHO diet group; VHCHO, very high-CHO diet group; Sed, sedentary group; Tr, exercise training group.
Several studies have shown that long-term intake of a high-CHO diet (~60% energy from CHO) resulted in an increase in pancreatic amylase activity (11–14). However, most studies have compared diets with largely different compositions (e.g., high-CHO diet vs. normal diet or high-fat diet), and it therefore remained unclear whether feeding a very-high CHO diet containing more CHO (~76% CHO) would also further increase pancreatic amylase activity. In the present study, the VHCHO group consumed significantly more CHO compared with the HCHO group (Table 1), and the pancreatic amylase activity was also greater in the VHCHO group than that in the HCHO group (Fig. 1). These results suggest that the higher CHO intake, the greater adaptation of the pancreatic digestive capacity obtainable. Furthermore, a very-high CHO diet intake when combined with endurance training, induced an increase in pancreatic amylase activity. This result also suggests the possibility that a very-high CHO diet and long-term endurance exercise training interact and synergistically upregulate the capacity for CHO digestion.

Muscle glycogen is an important energy substrate during prolonged strenuous exercise (3). Recently, we reported that CHO digestive capacity may be one of the determinant factors for muscle glycogen content because pancreatic amylase activity was highly and positively correlated with muscle glycogen concentration in untrained and trained rats (10). In the present study, the highest muscle glycogen level was observed in the VHCHO-Tr group (Fig. 3B), which had the highest pancreatic amylase activity (Fig. 1). Furthermore, a significantly strong association was found between pancreatic amylase activity and glycogen concentration in the epitrochlearis muscle (Fig. 3D). These results support the notion mentioned above and suggest that the combination of a very-high CHO diet and endurance training induced higher muscle glycogen levels (Fig. 3B), possibly through the enhanced CHO digestive capacity by promoting glucose delivery to muscle. Because we could not, of course, rule out the possibility that this is not a causal relationship, future studies will be required to provide direct evidence proving this possibility.

It has been well documented that skeletal muscles that have adapted to endurance exercise with an increase in GLUT4, can transport blood glucose into the muscle and accumulate more muscle glycogen (27, 28). While this study also found training-induced muscle GLUT4 adaptation, interestingly, an intake of a very high-CHO diet additively increased both GLUT4 content and glycogen levels in the epitrochlearis muscle (Fig. 3A, B). Taken together, our results suggest that a very high-CHO diet intake, in combination with endurance training, substantially elevated muscle glycogen concentration via both enhanced muscle glucose transport capacity and pancreatic CHO digestive capacity. For Kenyan long-distance runners who have an extremely high daily CHO intake, adaptations in pancreas as well as skeletal muscle may contribute, at least in part, to their superior performance.

Since 90% of glucose absorption in the small intestine is mediated by SGLT1 and GLUT2 (16), the absorptive capacities of CHO can be determined by these transporters (15). The present study showed that feeding rats a very high-CHO diet for 4 wk did not affect intestinal glucose transporter contents (Fig. 2), whereas previous studies have reported that a high-CHO diet increases the density and/or activity of SGLT1, allowing greater CHO absorption (17, 18). The reasons for this difference in the results between the present and previous studies are unclear, but this could be due to the amount of CHO contained in the diets. Moran et al. (17) evaluated mRNA expression and protein content of SGLT1 and glucose uptake function in the small intestine when the proportion of CHO contained in the diet was 35.9, 52.6, and 60.3%, respectively. As a result, although the SGLT1 mRNA and protein levels and glucose uptake function in the intestine of animals fed a 52.6 and 60.3% CHO diet were significantly higher than that seen in the 35.9% CHO-fed group, there was no significant difference in these parameters in the intestines of animals fed either the 52.6 or 60.3% CHO diet. In our study, the rats were fed a diet containing 59% (HCHO group) or 76% (VHCHO group) CHO. It thus seems possible that SGLT1 expression was sufficiently increased and already saturated by feeding with the high-CHO diet (HCHO group), and no further increase was observed even after feeding the very high-CHO diet (VHCHO group). In addition, this study did not detect significant differences in the SGLT1 and GLUT2 content between the Sed and Tr groups after 4-wk endurance exercise training, suggesting that 4 wk of endurance training was not sufficient to cause any change in glucose transporters or pancreatic amylase activity. On the other hand, in our previous study (10), which reported an increase in intestinal SGLT1 content with 6 wk of endurance exercise training, the trained and untrained groups had similar total energy intakes. However, the present study has shown that the Tr group has lower total energy intake than the Sed group (Table 1), suggesting that this lower energy intake may result in little change in intestinal SGLT1 content. Endurance athletes generally have higher energy intake than non-athletes. Thus, future research will be required to examine the relationship between energy intake during endurance exercise training and intestinal glucose transporter contents. As another possibility, the lack of main effects and interaction for some parameters, including SGLT1 and GLUT2, might be due to the relatively small statistical power. Because this study analyzed the samples which have already been taken in our previous experiment (8), we could not change the sample size. Thus, it seems necessary to carefully consider the sample size before conducting a future study to clarify this point.

In conclusion, the results of this study show that intake of a very high-CHO diet in combination with long-term endurance exercise training synergistically increases the digestive capacity of CHO in the pancreas. This pancreatic adaptation may partially contribute to
the higher muscle glycogen content and has potential benefits for athletes engaged in high-intensity prolonged endurance exercise, such as those participating in a marathon race.

Authorship
Research conception and design: SK, TK, and ST; experiments: SK, TK, AF, AK, and MT; statistical analysis of the data: SK and TK; interpretation of the data: SK, TK, and ST; writing of the manuscript: SK, TK, and ST.

Saki Kondo and Takuya Karasawa contributed equally to this work.

Disclosure of state of COI
No conflicts of interest to be declared.

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