Pre-Exercise High-Fat Diet for 3 Days Affects Post-Exercise Skeletal Muscle Glycogen Repletion

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Summary Previous studies have shown that the short-term intake of a high-fat diet (HFD) impairs glucose metabolism. In this study, we investigated the influences of pre-exercise HFD intake for 3 d on post-exercise glycogen repletion in skeletal muscle in ICR mice. Mice received either an HFD (57% kcal from fat, 23% kcal from carbohydrate; HFD group) or standard laboratory chow (13% kcal from fat, 60% kcal from carbohydrate; Con group) for 3 d before exercise. Mice performed treadmill running at 25 m/min for 60 min and were orally administered a glucose (2 mg/g body weight) solution immediately after and at 60 min after exercise. A negative main effect of pre-exercise HFD intake was observed for skeletal muscle glycogen concentration from the pre-exercise phase to 120 min of post-exercise recovery (p<0.01). Blood glucose concentration in the HFD group was significantly higher than in the Con group at 120 min after exercise (p<0.01). No significant difference was observed in plasma insulin concentration. There were no significant between-group differences in the phosphorylation state of Akt Thr308, AMPK Thr172, AS160 Thr642, or glycogen synthase Ser641 or in glucose transporter 4 protein levels during post-exercise recovery. Our results suggest that the intake of a pre-exercise HFD for 3 d affects post-exercise glycogen repletion in skeletal muscle without impairing the insulin signaling cascade.

Key Words skeletal muscle, post-exercise recovery, high-fat diet, glucose

The pre-exercise glycogen store in skeletal muscle is considered a major determinant of exercise performance during moderate- to high-intensity exercise (1–4). When exercise intensity exceeds around 60–65% VO2max, the skeletal muscle glycogen store is the predominant source of energy (5, 6). Moreover, recent studies suggest that glycogen per se is important for skeletal muscle force production by the modulating excitation-contraction coupling process (7). When individuals are involved in multiple training sessions and competitions over successive days or within a day, enhancing glycogen repletion in skeletal muscle after exercise is essential for improving performance during subsequent exercise.

Some athletes alter their dietary composition of carbohydrate and fat for several days to enhance their exercise performance. For example, adaptation to a ketogenic diet, which severely restricts carbohydrate intake (<20 g/d or 5% of total daily energy intake) and contains high fat levels and an adequate amount of protein, has been shown to be useful for weight control without detrimental effects on skeletal muscle mass, power output, or strength after not only long-term (>2 wk) but also short-term (7 d) administration (8–10). Adaptation to a ketogenic diet increases fat utilization and reduces carbohydrate reliance during exercise without impairing endurance capacity at 62–64% VO2max under a state of insufficient carbohydrate availability (overnight fasting) (11). However, as a ketogenic diet sometimes causes side effects, including increased perceived exertion during exercise (12), decreased training load (13), and lower cognitive performance (14), it may not be a practical approach. Instead of a ketogenic diet, consuming a low but not severely restricted amount of carbohydrate and a high amount of fat for 3–10 d (fat adaptation) is also beneficial for increasing fat utilization during exercise (15–17). This fat adaptation is usually followed by 1–3 d of a high-carbohydrate diet to restore muscle glycogen stores before competition. However, whether the short-term adaptation to high-fat diet (HFD) influences glycogen repletion in skeletal muscle after exercise has not been elucidated. Previous studies showed that HFD feeding for a longer period (2 or 4 wk) impairs insulin-stimulated glucose uptake in skeletal muscle, which is a major rate-limiting step for glycogen synthesis, after endurance exercise in rats (18, 19). In addition, HFD intake for 2–5 d attenuates blood glucose disposal during a glucose tolerance test (20, 21) and insulin sensitivity during a hyperinsulinemic-euglycemic glucose clamp (22, 23) in both humans and rodents, while some studies reported that HFD intake for 3 d does not affect glucose uptake in skeletal muscle at rest (24, 25). Furthermore, although enhanced insulin sensitivity in skeletal muscle and the whole body by physical exercise is generally thought to persist for 24–48 h, a single bout of exercise following a 3 d HFD does not improve whole-body insulin sensitivity (26). Even though enhanced glucose uptake and glycogen resynthesis in skeletal muscle are observed immediately after exercise, the pos-
sibility exists that pre-exercise short-term exposure to an HFD may attenuate post-exercise glycogen repletion in skeletal muscle. In this study, we investigated whether the consumption of a pre-exercise HFD for 3 d influences post-exercise skeletal muscle glycogen recovery.

METHODS

Animals. Six-week-old male ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed in a room maintained at 23°C with 3 mice per cage, and were acclimatized for 1 wk. The mice were given free access to a standard chow (MF: 3.6 kcal/g, 60% kcal from carbohydrate, 13% kcal from fat, 27% kcal from protein; Oriental Yeast Co., Ltd., Tokyo, Japan). All procedures performed in this study involving animals were in accordance with the ethical standards of the Committee on Animal Care and Use, The University of Tokyo, and all protocols of research on animals were approved by this committee (approval number: 24-4).

Experimental procedure. We set the dark phase to 09:00–21:00, and all experimental treatments were performed in the dark phase when the mice were active. At 3 d before the experimental day, all mice were familiarized with treadmill exercise at a speed of 25 m/min for 10 min. The mice were divided into an HFD group (n = 5–7) and a control (Con) group (n = 5–7) with similar mean body weights. The mice in the HFD group received the HFD (HFD32; 5.1 kcal/g, 23% kcal from carbohydrate, 57% kcal from fat, 20% kcal from protein; CLEA Japan) for 3 d; the mice in the Con group received the standard chow. On the day of the experiments, after the mice were fasted for 1 h to avoid a postprandial state, they ran on the treadmill at 25 m/min for 60 min. All mice were orally administered 2 mg/g body weight glucose dissolved in water immediately after exercise and at 60 min after exercise. The mice were then housed individually in standard cages. The mice were sacrificed by cervical dislocation immediately after exercise or at 60 min after exercise. The mice were then housed individually in standard cages. The mice were sacrificed by cervical dislocation immediately after exercise or at 60 or 120 min after exercise. In addition, to measure baseline levels of each metabolite, the mice were sacrificed at rest after fasting for 1 h. Blood was taken from the open chest. Plasma, liver, and skeletal muscles of the lower hind limb were frozen in liquid nitrogen and stored at −80°C. Blood samples were collected into capillary tubes from the tail vein before glucose administration (0 min) and at 20, 60 (immediately before the second glucose administration), 80, and 120 min after the first glucose administration. We also collected blood samples into capillary tubes from the tail of the mice sacrificed for baseline measurement. The capillary tubes were centrifuged and plasma samples were stored at −80°C.

Analytical methods. Blood glucose concentrations were measured using an auto-analyzer (Gluestet Ace; Arkray, Inc., Kyoto, Japan). Plasma free fatty acid (FFA) concentrations were measured using a Wako NEFA C Test Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Muscle and liver glycogen concentrations were measured using the phenol-sulfuric acid method (27). Plasma insulin concentrations were measured using a Mouse Insulin ELISA Kit (Mercodia AB, Uppsala, Sweden).

Protein isolation and Western blotting. Protein isolation from the tibialis anterior muscle and Western blotting were performed as described previously (28). The tibialis anterior muscles were homogenized in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 10 mM sodium β-glycerol phosphate, 5 mM sodium pyrophosphate, 2 mM dithiothreitol, 1 mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride. 10 μg/mL each of aprotinin, leupeptin, and pepstatin A, pH 7.5). After centrifugation at 2,000 × g for 15 min at 4°C, the supernatants were collected and their protein concentrations were determined by the Bradford assay (Quick Start™ Bradford Dye Reagent 1×; Bio-Rad, Hercules, CA). The supernatants were diluted with buffer A (1 mM EDTA, 10 mM 2-amino-2-hydroxymethyl-1,3-propanediol, pH 7.4). For Western blotting, proteins (10 μg) and pre-stained molecular weight markers (BioDynamics Laboratory, Inc., Tokyo, Japan) were run on 12% SDS-PAGE gels for 60 min at 150 V. The proteins were then transferred from the gels to Hybond-P polyvinylidene difluoride transfer membranes (GE Healthcare Japan, Tokyo, Japan) for 75 min at 100 V. The membranes were blocked with TBS-T (20 mM Tris base, 137 mM NaCl, 0.1 mM HCl, 0.1% [vol/vol] Tween 20, pH 7.5) containing 5% (w/v) skim milk or 3% (w/v) bovine serum albumin for 60 min at room temperature. The membranes were incubated with the primary antibody against glucose transporter 4 (GLUT4, 07-1404; Millipore, Temecula, CA), glycolen synthase (GS, #3893; Cell Signaling Technology [CST] Japan, Tokyo, Japan), phosphorylated GS (Ser641, #3891; CST), Akt (#9272; CST), phosphorylated Akt (Thr308, #9275; CST), AMP-activated protein kinase (AMPK, #2532; CST), phosphorylated AMPK (Thr172, #2513; CST) and phosphorylated AS160 (Thr642, #4288; CST) in TBS-T (1 : 4,000 dilution) overnight at 4°C. Subsequently, the membranes were incubated for 60 min at room temperature with goat-anti-rabbit IgG (American Qualex, San Clemente, CA) in TBS-T (1 : 4,000 dilution). Antibodies were visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA) and detected using the ChemiDoc system (Bio-Rad). Densitometric analyses of the captured images were performed using Bio-Rad Quantity One software version 4.6.1. All membranes were stained with Ponceau-S solution (P7170–1L; Sigma-Aldrich, St. Louis, MO) to ensure that the proteins were loaded equally.

Statistical analysis. All values are expressed as the mean ± standard error. We used Prism 5 software (GraphPad Software, San Diego, CA) for the analyses. Two-way analysis of variance (pre-exercise dietary composition × time) was performed to determine differences in the levels of skeletal muscle and liver glycogen and plasma FFA. If there was an interaction, the Tukey-Kramer multiple-comparison test was performed. We used two-way repeated-measures analysis of variance (pre-exercise dietary composition × time) to determine differences in body weight, food consumption and blood.
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Fig. 1. Glycogen concentration in the tibialis anterior muscle (A) and liver (B) of mice that consumed the high-fat diet (HFD group; black) or standard diet (Con group; white) before exercise. Values are means±standard error. n=6 per group. **p<0.01 vs. the Con group at the same time. †p<0.05 and ††p<0.01 vs. baseline. n.s.: not significant.

Table 1. Body weight and food consumption of mice.

<table>
<thead>
<tr>
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<th>Pre-intervention</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3 (before exercise)</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td>Con group</td>
<td>35.3±0.6</td>
<td>35.0±0.6</td>
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<tr>
<td></td>
<td></td>
<td>HFD group</td>
<td>35.5±0.4</td>
<td>35.5±0.5</td>
</tr>
<tr>
<td>Food consumption (kcal)</td>
<td></td>
<td>Con group</td>
<td>16.1±0.6</td>
<td>17.9±0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HFD group</td>
<td>25.5±0.9**</td>
<td>21.7±0.8**</td>
</tr>
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</table>

Values are means±standard error. n=24 or 25 per group. **p<0.01 vs. the Con group at the same time point.

glucose and plasma insulin levels. If there was an interaction, Bonferroni’s multiple comparisons test was performed for comparisons between two groups at the same time point. We used an unpaired t-test to determine differences in protein levels in skeletal muscle between the two groups. Statistical significance was set at p<0.05.

RESULTS

Food consumption and body weight

We did not observe any significant main effect of pre-exercise dietary composition in body weight (Table 1). We observed a significant interaction (pre-exercise dietary composition×time) in food consumption (p<0.01). On the first and second day, food consumptions of the HFD group were significantly higher than those of the Con group (p<0.01). On the third day, no significant difference was observed in food consumption between the two groups.

Glycogen levels in skeletal muscle and liver

No significant interaction (pre-exercise dietary composition×time) was observed in skeletal muscle glycogen concentration (Fig. 1A). There was a negative main effect of pre-exercise HFD intake on skeletal muscle glycogen concentration (p<0.01). The rates of skeletal muscle glycogen accumulation were 2.2 mg/g wet weight (wt) in the HFD group and 3.1 mg/g wt in the Con group during the first 60 min; and 2.3 mg/g wt in the HFD group and 3.8 mg/g wt in the Con group during the second 60 min of post-exercise recovery. We also measured glycogen concentration of liver, which is a major site of glycogen storage, as well as skeletal muscles. There was a significant interaction (pre-exercise dietary composition×time) in liver glycogen concentration (p<0.01, Fig. 1B). Glycogen levels in the liver were significantly lower in the HFD group than in the Con group at baseline (p<0.01). There were no significant
differences in liver glycogen concentration between the two groups at the end of exercise, or at 60 or 120 min after exercise. In the HFD group, liver glycogen concentration at 60 min after exercise was not significantly different from the baseline level, while liver glycogen concentration at 60 min after exercise in the Con group was lower than the baseline level (p<0.05).

Plasma FFA levels

As some studies showed that an increase in blood FFA levels leads to a decrease in glycogen synthesis in skeletal muscle (29–31), we determined whether a pre-exercise HFD for 3 d affected FFA levels. No significant interaction (pre-exercise dietary composition×time) was observed in plasma FFA levels. Plasma FFA levels were not affected by 3 d of pre-exercise dietary intervention (baseline: 0.33±0.02 mEq/L in the HFD group vs. 0.31±0.03 mEq/L in the Con group; at the end of exercise: 0.49±0.05 mEq/L in the HFD group vs. 0.47±0.03 mEq/L in the Con group; at 60 min after exercise: 0.45±0.03 mEq/L in the HFD group vs. 0.44±0.07 mEq/L in the Con group; and at 120 min after exercise: 0.37±0.04 mEq/L in the HFD group vs. 0.41±0.04 mEq/L in the Con group).

Glucose and insulin levels in blood after glucose administration

At baseline, no significant differences were observed in either blood glucose levels (6.2±0.4 mmol/L in the HFD group vs. 5.6±0.4 mmol/L in the Con group) or plasma insulin levels (3.5±1.0 ng/mL in the HFD group vs. 2.6±0.4 ng/mL in the Con group). During post-exercise recovery, a significant interaction (pre-exercise dietary composition×time) was observed in blood glucose levels (Fig. 2A). At the end of exercise, no significant difference in blood glucose levels was observed between the two groups. There was no significant difference in blood glucose levels for 60 min after the first administration of post-exercise oral glucose. At 120 min after exercise (60 min after the second administration of post-exercise oral glucose), blood glucose levels were significantly higher in the HFD group than in the Con group (p<0.01). There was no significant difference in plasma insulin levels between the two groups during post-exercise recovery (Fig. 2B).

Levels and phosphorylated states of proteins involved in insulin signaling

Insulin resistance in skeletal muscle induced by an
HFD is associated with lower phosphorylated levels of the insulin signaling cascade (19, 32, 33); conflicting results were observed in post-exercise skeletal muscle of mice which were provided HFD for 2 or 4 wk (18, 34). We determined total amounts and phosphorylation states of proteins which were involved in glucose uptake and glycogen synthesis after post-exercise glucose administration. There were no significant between-group differences in the phosphorylation state of Akt Thr308, AMPK Thr172, AS160 Thr642, or GS Ser641 either at 60 min (Fig. 3A) or at 120 min (Fig. 3B) after exercise. No significant between-group difference in GLUT4 protein level was observed at any time point (at 60 min: 94.6±7.1 arbitrary unit (AU) in the HFD group vs. 100±10.4 AU in the Con group; at 120 min: 95.8±5.5 AU in the HFD group vs. 100±4.4 AU in the Con group).

**DISCUSSION**

In this study, we determined the effects of pre-exercise meal composition on skeletal muscle glycogen recovery by providing ICR mice an HFD with compensatory low carbohydrate (57% kcal from fat, 23% kcal carbohydrate) or standard laboratory chow (13% kcal from fat, 60% kcal from carbohydrate) for 3 d before endurance exercise. We used HFD32 chow as the diet for the HFD group, because in some previous studies investigating either the effect of fat adaptation or attenuation of carbohydrate metabolism induced by HFD, subjects consumed high-fat and low-carbohydrate diet having a similar ratio of carbohydrate (around 20% kcal) and/or fat (around 60% kcal) (16, 18–20, 24, 26, 34). As previous studies suggested that HFD intake for 2–5 d attenuates blood glucose disposal during a glucose tolerance test (20, 21) and insulin sensitivity during a hyperinsulinemic-euglycemic glucose clamp (22, 23), even if the subjects performed exercise before the test (26, 34), we hypothesized that HFD intake for 3 d would attenuate post-exercise skeletal muscle glycogen recovery. In the present study, mice consuming the HFD for 3 d showed: (1) lower skeletal muscle glycogen levels from pre-exercise to 120 min after exercise, even though they were provided with an adequate amount of glucose during post-exercise recovery; and (2) higher blood glucose levels at 120 min after the first administration of post-exercise oral glucose without impairing the insulin signaling pathway in skeletal muscle during post-exercise recovery.
On the first and the second days, food consumptions of the HFD group were significantly higher than those of the Con group. We could not conclude whether food intake was increased by the change from standard lab chow to HFD because we did not measure the food consumption before the intervention. However, there was no significant difference in food consumption between the two groups on the third day. Furthermore, we did not observe any significant difference in body weight between the two groups during pre-exercise dietary intervention. Therefore, differences in food consumptions on the first and the second days between the two groups might not influence other results in the present study.

Previous in vitro studies showed that 2 or 4 wk of HFD feeding impairs insulin-stimulated glucose uptake in skeletal muscle after endurance exercise in rats (18, 19). We found that blood glucose levels at 120 min after exercise were significantly higher in the HFD group than in the Con group. The majority of glucose in blood is taken up by skeletal muscle. After endurance exercise, 66% of glucose deprived from splanchnic output after oral glucose administration is incorporated to glycogen in skeletal muscle, while the brain utilizes 17% of glucose and other tissues utilize 6% (35). Therefore, the decrease in blood glucose levels after post-exercise glucose administration is mainly influenced by glucose uptake in skeletal muscle. While previous studies showed that consuming an HFD for 3 d did not influence glucose uptake in skeletal muscle at rest (24, 25), our results suggest that HFD intake for 3 d before exercise attenuates glucose uptake in skeletal muscle during the post-exercise phase.

Previous studies, which showed that HFD feeding for 2 or 4 wk impairs insulin-stimulated glucose uptake in skeletal muscle, did not measure glycogen levels in the fed state during post-exercise recovery (18, 19). As it should be usual that subjects consume carbohydrates after exercise to stimulate glycogen recovery, we provided the mice with a glucose solution orally during their recovery from endurance exercise. We found a negative main effect of the pre-exercise HFD on skeletal muscle glycogen concentration from pre-exercise to 120 min of post-exercise recovery. This is the first study to show that the intake of an HFD for only 3 d affects skeletal muscle glycogen levels throughout the pre-exercise and post-exercise phases. Post-exercise glucose uptake in skeletal muscle is activated in an insulin-independent manner (contraction per se) and an insulin-dependent manner. A previous study showed that 2 wk of HFD intake did not affect the stimulation of glucose uptake in skeletal muscle induced by exercise with no insulin condition (19). Therefore, it is possible that pre-exercise HFD consumption for 3 d did not affect insulin-independent but only insulin-dependent glucose uptake during post-exercise recovery. In general, glucose uptake and glycogen synthesis of skeletal muscle stimulated by insulin and/or contraction are enhanced when glycogen levels are low (36–38). Therefore, the skeletal muscle glycogen levels in the HFD group may have reached those of the Con group during a later phase of post-exercise recovery with oral glucose administration. We provided the second glucose solution at 60 min after the exercise and investigated whether the glycogen level in the HFD group reached the level of the Con group. However, the HFD group maintained lower skeletal muscle glycogen levels throughout the 120 min of post-exercise recovery. This result is consistent with the evidence that diabetic subjects have lower glycogen synthesis rates in skeletal muscle both at rest (39) and after exercise (40), even though their basal glycogen levels are lower than those of non-diabetic subjects. Although endurance exercise itself should stimulate glucose uptake and glycogen synthesis in skeletal muscle, pre-exercise HFD intake might interfere with glycogen repletion during post-exercise recovery.

At 60 min after exercise, the liver glycogen concentration in the HFD group recovered to its baseline level, while it was still significantly lower than the baseline level in the Con group. As mentioned above, the majority of exogenous glucose is utilized for skeletal muscle glycogen repletion during the post-exercise phase (35). Only 9% of infused glucose is reportedly used for hepatic glycogen synthesis after exhaustive exercise in healthy rats (41). A major fraction of liver glycogen is thought to be formed via gluconeogenesis (42). Previous studies indicated that HFD intake for 7–14 d enhances gluconeogenesis in the liver after an overnight fast (43, 44). In the present study, liver glycogen concentration of the HFD group at baseline was significantly lower than that of the Con group. It might be due to the lower intake of carbohydrate in the HFD group. In addition, gluconeogenesis seemed not to be stimulated so much because food was withheld for only 1 h before the sampling. However, it is possible that pre-exercise HFD intake for 3 d stimulated gluconeogenesis in the liver when the glycogen level was decreased by endurance exercise. Furthermore, higher gluconeogenesis in the liver induced by pre-exercise HFD intake might account for the higher blood glucose level after post-exercise glucose administration. This hypothesis is supported by a previous study which showed the intake of HFD for 3 d resulted in higher hepatic glucose output during the euglycemic hyperinsulinemic clamp test (24).

While some studies reported that rodents fed an HFD for 3 d do not have impaired glucose uptake in skeletal muscle at rest (24, 25), we found that HFD intake for 3 d had significant negative effects on skeletal muscle glycogen and blood glucose levels after post-exercise glucose administration. As it has been shown that consumption of an HFD for only 1 d attenuates carbohydrate oxidation (45, 46), a reduction of carbohydrate metabolism in skeletal muscle could occur after 1 or several days of HFD intake. However, the mechanisms by which short-term HFD intake attenuates glucose uptake and glycogen synthesis in skeletal muscle have not been elucidated. An acute increase in blood FFA levels leads to a decrease in glucose uptake and glycogen synthesis in skeletal muscle (29–31). However, the development of insulin resistance induced by short-term (~1 wk) HFD con-
sumption is not always concomitant with the increase in blood FFA levels (34, 47). In the present study, we did not find any increase in plasma FFA levels following 3 d of HFD intake either at rest or during the post-exercise recovery phase; however, it cannot be ruled out that the increased FFA levels were offset by enhanced utilization of FFAs as a fuel following adaptation to the HFD. Some previous studies reported that insulin resistance in skeletal muscle induced by an HFD is associated with lower phosphorylated levels of the insulin signaling cascade (19, 32, 33). However, others showed that the lower glucose uptake in post-exercise skeletal muscle after HFD consumption is not always accompanied by an impairment of the insulin signaling cascade (18, 34). The results of the present study indicate that pre-exercise HFD intake for 3 d did not impair the insulin signaling cascade during the post-exercise phase recovery with carbohydrate administration. The mechanism by which HFD intake for 3 d attenuates post-exercise glycogen repletion in skeletal muscle remains unclear.

In conclusion, we observed a significant negative effect of the intake of a pre-exercise HFD with compensatory low carbohydrates for only 3 d on skeletal muscle glycogen concentration from pre-exercise to 120 min of the post-exercise phase, although both groups were provided with a similar amount of glucose during the post-exercise phase. As the blood glucose level after oral glucose administration was higher in the pre-exercise HFD intake group than in the Con group, it is possible that glucose uptake in skeletal muscle after post-exercise glucose ingestion was affected by pre-exercise meal composition.

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