Athletes metabolize a massive amount of calories derived from endogenous fuel such as glycogen in muscle and liver during prolonged strenuous exercise. Because these endogenous fuels are limited in quantity, an exogenous calorie supply is needed during endurance exercise. Therefore, calorie intake is one of the key strategies to replace depleted glycogen sources during endurance exercise. Athletes require an efficient energy supplement to enhance and maintain their performance and prevent fatigue at later stages of exercise.

Carbohydrate (CHO) supplementation is known to maintain physical activity performance by providing energy to muscles (1). CHO oxidation in fast muscles is a more useful method of energy utilization than fat oxidation during high-intensity exercise (2). In particular, many reports suggest that CHO supplementation maintains performance during prolonged strenuous exercise (1, 3–5). A progressive shift from muscle glycogen to blood glucose occurs with an increase in exercise duration. Coyle et al. (6) reported that blood glucose levels decrease to those seen in hypoglycemia in well-trained cyclists after 3 h of exercise. That study also reported that CHO supplementation during exercise suppresses hypoglycemia and delays fatigue, although the muscle glycogen content was not restored, suggesting the importance of maintaining blood glucose levels during long-term exercise.

Blood glucose levels are maintained by glycogenolysis and gluconeogenesis, which primarily occur in the liver. Hepatic glycogen is digested and reduced during long-term exercise to supply glucose. In addition, gluconeogenesis is activated by exercise (8). Alanine (Ala) is a protein-derived gluconeogenic precursor (9). During exercise and fasting, blood Ala levels derived from muscle protein breakdown increase considerably and are converted to glucose in the liver (10). Ala supplementation gradually increases blood glucose levels (11). Proline (Pro) is also a gluconeogenic amino acid and a major glucose production source in the liver. Therefore,
we hypothesized that combined supplementation of gluconeogenic substrates and CHO may be more effective in maintaining blood glucose levels and enhancing exercise performance than CHO supplementation alone. Thus, we investigated the beneficial effects of a single supplementation of CHO combined with Ala and Pro (CHO+AlaPro) on the maintenance of blood glucose levels and exercise endurance performance in mice.

MATERIALS AND METHODS

Materials. Maltodextrin was used as the CHO source, since it has been preferentially utilized as a supplement by athletes. Maltodextrin (TK-16) was purchased from Matsutani Chemical Industry (Hyogo, Japan). L-Ala (L-Ala : D-Ala was approximately 1 : 1), which is widely used as a food additive for humans and animals, and L-Pro were obtained from Ajinomoto Co., Inc. (Tokyo, Japan).

Male C57BL/6J mice were obtained fromCLEA Japan, Inc. (Tokyo, Japan) and housed at 22.5 ± 1°C under a 12 : 12 h light/dark cycle. Diet chow (CRF-1, Charles River Laboratories Japan, Inc., Kanagawa, Japan) and tap water were provided ad libitum. All animal procedures were approved by the Committee for Animal Experiments at Ajinomoto and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Blood glucose measurements during wheel running. To acclimate mice to an exercise wheel (diameter = 40 cm; Oriental Motor Co., Ltd., Tokyo, Japan), they were trained to run for 1 h daily, 3 d per week. After overnight fasting, mice were orally administered either CHO alone (maltodextrin, 1.25 g/kg) or CHO (maltodextrin, 1.0 g/kg) supplemented with dl-Ala (0.225 g/kg) and l-Pro (0.0225 g/kg). The ratio of Ala to Pro was determined based on the ratio of l-Ala flux to l-Pro flux in the body (12, 13). Fifteen minutes after administration, mice ran at 10.5 m/min for 170 min. Blood samples were collected from the tail vein before administration and at 65, 100, 135, and 170 min after beginning exercise. Blood glucose was measured using a glucose sensor (GT-1820, Arkray, Kyoto, Japan).

Glycogen and plasma glucagon measurements. After overnight fasting, mice were orally administered either CHO alone (maltodextrin, 1.25 or 1.0 g/kg) or CHO (maltodextrin, 1.0 g/kg) supplemented with dl-Ala (0.225 g/kg) and l-Pro (0.0225 g/kg). Fifteen minutes after administration, mice ran at 10.5 m/min for 60 or 90 min. Liver, gastrocnemius muscle, and blood samples were collected after decapitation, and the glycogen content was determined as described previously (14). Plasma glucagon levels were measured using an enzyme immunoassay kit (YK090, Yanaihara Institute Inc., Shizuoka, Japan).

Endurance performance test. Mice were acclimated to a one-lane treadmill (Arco Systems, Chiba, Japan) for 2 d before the pre-endurance test by running for 60 min at 14 m/min. On the day of the pre-endurance test, mice were orally administered water after overnight fasting. Fifteen minutes after administration, mice ran for 60 min at 14 m/min followed by an increase in speed of 2 m/min for each additional 5 min until exhaustion. The maximum speed for each increment was 36 m/min, and if mice were able to reach this speed successfully, they were maintained at this speed until exhaustion. Mice were defined as exhausted after remaining on the shock grid for 5 continuous seconds, as described previously (15). One week after the pre-endurance test, mice were divided into two groups with equivalent average exhaustion times determined by the pre-endurance test. Overnight-fasted mice were orally administered either CHO alone (maltodextrin, 2.0 g/kg) or CHO (maltodextrin, 1.0 g/kg) supplemented with dl-Ala (0.9 g/kg) and l-Pro (0.1 g/kg). Fifteen minutes after administration, mice ran for 60 min at 14 m/min followed by an increase in speed of 2 m/min for each additional 5 min until exhaustion. Exhaustion time of mice was measured.

Statistical analysis. Data are presented as mean ± standard error. A two-way repeated-measures analysis of variance (ANOVA) was used to analyze blood glucose levels, with supplementation (CHO, CHO+amino acids) and various running time points as the independent variables. A two-way factorial ANOVA was used to analyze the glycogen content, glucagon level with supplementation (CHO, 1.0 g/kg; CHO, 1.25 g/kg; and CHO, 1.0 g/kg+amino acids), and running time points (60 and 90 min). The Bonferroni multiple comparisons test was applied to compare groups when appropriate. The unpaired Student’s t-test was used to compare endurance performance between the two groups. GraphPad Prism ver. 6.0 for Windows (GraphPad Software, San Diego, CA) was used for data analysis. Results were considered significant at p < 0.05.

RESULTS

Blood glucose levels

We examined whether a single supplementation of CHO+AlaPro could maintain blood glucose levels during long-term exercise. Figure 1 shows the time course of blood glucose levels during mice wheel running. Supplementation (p < 0.001) and time (p < 0.001) were found to have significant effects on blood glucose levels, with a significant interaction between these factors (p < 0.05). CHO+AlaPro supplementation resulted in significantly higher blood glucose levels 100 and 170 min after beginning exercise than CHO alone. This result indicates that a single supplementation of CHO+AlaPro significantly suppressed the decrease in blood glucose levels during the late stages of this exercise test compared to CHO alone.

Glycogen content

To determine the accumulation of glycogen in the liver and muscle, we used the same running wheel model with CHO alone and CHO+AlaPro supplementation (Fig. 2). Supplementation (p < 0.05) and time (p < 0.001) were found to have significant effects on hepatic glycogen, with no interaction between these factors (p = 0.07). The hepatic glycogen content of mice supplemented with CHO+AlaPro was significantly higher than CHO alone.
higher than that of mice supplemented with CHO alone (1.0 g/kg) after wheel running for 60 min. The hepatic glycogen content at 90 min was low and not significantly different among the three groups. No significant difference was observed in the muscle glycogen content among the three groups (Fig. 3).

**Endurance performance**

We measured running time until exhaustion to evaluate the effects of AlaPro on endurance performance. During pre-endurance tests, the mean running time of mice was 107.0 ± 1.6 min. Figure 5 shows the treadmill running time until exhaustion of mice supplemented CHO alone (113.3 ± 3.0 min) and those supplemented CHO + AlaPro at 60 or 90 min.

**Plasma glucagon levels**

We examined whether CHO + AlaPro supplementation increased plasma glucagon levels during long-term exercise compared to CHO alone. Figure 4 shows plasma glucagon levels during mice wheel running. No significant difference in plasma glucagon levels was found between mice supplemented CHO alone and those supplemented CHO + AlaPro. **Fig. 1**. The effect of combined supplementation of carbohydrate (CHO), alanine (Ala), and proline (Pro) on blood glucose levels during long-term exercise. Mice were orally administered CHO alone (1.25 g/kg) or CHO + AlaPro (CHO, 1.0 g/kg; Ala, 0.225 g/kg; and Pro, 0.0225 g/kg) 15 min before exercise. Blood glucose levels of mice were assessed before supplementation and 65, 105, 135, and 170 min after beginning exercise. Data represent mean ± standard error (n=18/group). *p<0.05.

**Fig. 2**. The effect of combined supplementation of carbohydrate (CHO), alanine (Ala), and proline (Pro) on the hepatic glycogen content during long-term exercise. Mice were orally administered CHO (1.0 g/kg), CHO (1.25 g/kg), or CHO + AlaPro (CHO, 1.0 g/kg; Ala, 0.225 g/kg; and Pro, 0.0225 g/kg) 15 min before exercise. The glycogen content in the liver of mice exercised for 60 and 90 min was assessed. Data represent mean ± standard error (n=18/group). *p<0.05.
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This result showed that supplementation of CHO alone. This result showed that supplementation of CHO + AlaPro enhanced endurance performance during treadmill running even with isocaloric intake between the two groups.

**DISCUSSION**

Energy supplementation, particularly with CHO, is very important during prolonged exercise because it maintains blood glucose levels during the later stages of exercise. However, athletes cannot freely ingest CHO at short intervals during competitions. It would be of benefit for many athletes if a single CHO ingestion before exercise could maintain blood glucose levels for a long period of time.

In this study, we found that combined supplementation of CHO, Ala, and Pro before exercise resulted in significantly higher blood glucose levels in mice than an equivalent amount of CHO alone from 100 min onward after beginning exercise. This finding suggests that combined supplementation of CHO, Ala, and Pro suppressed the decrease in blood glucose levels during endurance exercise even if they were ingested only once before the start of exercise. A single supplementation of CHO with these amino acids may be suitable for energy supplementation, rather than CHO alone, for several sports that have limited break times.

Ala promotes glucagon secretion (16). However, differences in plasma glucagon levels were not observed between mice supplemented with CHO alone and those supplemented with CHO, Ala, and Pro during running.
Thus, glucagon was considered not to contribute to the maintenance of blood glucose levels under our exercise conditions.

The hepatic glycogen content of mice supplemented with CHO, Ala, and Pro was higher than that of mice supplemented CHO alone, suggesting that Ala and Pro promote glycogen synthesis or preserve it in the liver during the early stage of endurance exercise. Ala is a gluconeogenic amino acid that is produced in muscles through protein breakdown and transamination, and is delivered to the liver via the blood circulation when energy is required during long-term exercise (17). Indeed, Ala level decreases in the blood during long-term exercise (18). One study suggested that exogenous Ala supplementation serves as an energy source during exercise (19). Sumida and Donovan (20) reported increases in maximal gluconeogenic flux from Ala after endurance training. Moreover, Pro promotes hepatic glycogen synthesis (21, 22). Souza et al. (23) reported that administration of a gluconeogenic precursor promotes better glycemic recovery than glucose itself. Ala and Pro provide a gluconeogenic amino acid “pool” that can be used as a glucose source, thus attenuating the decrease in blood glucose levels during long-term exercise.

Several studies have reported that the reduction in hepatic glycogen is greater than that in muscle glycogen in exercising rodents compared to those in exercising humans (24–26). For example, muscle glycogen stores are depleted 40–70%, whereas liver glycogen stores decrease 85% during less strenuous exercise regimens (26). In the present study, no difference was observed between muscle glycogen stores of mice exercised for 60 and 90 min, whereas liver glycogen stores of mice exercised for 90 min decreased more than those of mice exercised for 60 min. This result is consistent with the observation that muscle glycogen stores have relatively little importance in endurance exercise in mice compared to liver glycogen stores. Although the muscle glycogen content remained unchanged on combined supplementation of CHO, Ala, and Pro in this rodent model, further studies are needed to determine the effect of Ala and Pro on muscle glycogen in humans because the importance of adequate muscle glycogen to sustain exercise in humans has been well documented.

Energy from CHO oxidation is required to perform prolonged exercise. The exhaustion time of the mice ingesting CHO alone was significantly longer than that of mice ingesting water. This result shows that CHO is a useful supplementation for endurance performance. Administration of Ala and Pro with CHO further prolonged running time until exhaustion compared to CHO alone. This result was consistent with our hypothesis that Ala and Pro enhance endurance performance during long-term exercise. We found that combined supplementation of CHO, Ala, and Pro was better than CHO alone during long-term exercise. Klein et al. (27) reported the effects of Ala and CHO supplementation on the performance of cyclists during short-term exercise. They found no significant difference in performance at the end of endurance exercise rounds (45 min at 75% VO₂ max) between cyclists who received a placebo and those that received both Ala and sugar supplements. The energy source progressively shifts from muscle glycogen to blood glucose with an increase in exercise duration (6). Therefore, blood glucose is more critical for endurance performance during the later stages of long-term exercise (28). The supplementation of gluconeogenic amino acids with CHO would support endurance performance during more long-term exercise rather than short-term exercise because this mixture is useful when blood glucose levels decrease from prolonged exercise. The effects of Ala and Pro on endurance performance need further confirmation in human studies of long-term exercise.

In conclusion, we demonstrated that combined supplementation of Ala, Pro, and CHO maintained blood glucose levels during the later stages of exercise and improved endurance performance compared to supplementation of isocaloric CHO alone. The higher hepatic glycogen content induced by supplementation with Ala and Pro may have contributed to maintaining blood glucose levels and endurance performance. These results suggest that CHO supplementation with Ala and Pro may be a more effective strategy for energy intake during long-term exercise than CHO alone.

Acknowledgments
We would like to thank Natsuki Nishikawa and Shiori Takahashi for their technical assistance.

REFERENCES

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