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[1]*Doctoral Program in Sports Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tennodai, Tsukuba, Ibaraki, Japan; and* [2]*Health Care Food Research Laboratories, Kao Corporation, Bunka, Sumida, Tokyo, Japan*

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Iwayama K, Kawabuchi R, Park I, Kurihara R, Kobayashi M, Hibi M, Oishi S, Yasunaga K, Ogata H, Nabekura Y, Tokuyama K. Transient energy deficit induced by exercise increases 24-h fat oxidation in young trained men. *J Appl Physiol* 118: 80–85, 2015. First published November 13, 2014; doi:10.1152/japplphysiol.00697.2014.—Whole body fat oxidation increases during exercise. However, 24-h fat oxidation on a day with exercise often remains similar to that of sedentary day, when energy intake is increased to achieve an energy-balanced condition. The present study aimed to examine a possibility that time of the day when exercise is performed makes differences in 24-h fat oxidation. As a potential mechanism of exercise affecting 24-h fat oxidation, its relation to exercise-induced transient energy deficit was examined. Nine young male endurance athletes underwent three trials of indirect calorimetry using a metabolic chamber, in which they performed a session of 100 min of exercise before breakfast (AM), after lunch (PM), or two sessions of 50 min of exercise before breakfast and after lunch (AM/PM) at 65% of maximal oxygen uptake. Experimental meals were designed to achieve individual energy balance. Twenty-four-hour energy expenditure was similar among the trials, but 24-h fat oxidation was 1,142 ± 97, 809 ± 88, and 608 ± 46 kcal/24 h in descending order of its magnitude for AM, AM/PM, and PM, respectively ($P < 0.05$). Twenty-four-hour carbohydrate oxidation was 2,558 ± 110, 2,374 ± 114, and 2,062 ± 96 kcal/24 h for PM, AM/PM, and AM, respectively. In spite of energy-balanced condition over 24 h, exercise induced a transient energy deficit, the magnitude of which was negatively correlated with 24-h fat oxidation ($r = -0.72$, $P < 0.01$). Similarly, transient carbohydrate deficit after exercise was negatively correlated with 24-h fat oxidation ($r = -0.40$, $P < 0.05$). The time of the day when exercise is performed affects 24-h fat oxidation, and the transient energy/carbohydrate deficit after exercise is implied as a factor affecting 24-h fat oxidation.

energy balance; energy flux; epoc; metabolic chamber

WHOLE BODY FAT OXIDATION increases during endurance exercise, and the increased fat oxidation persists during the postexercise period (4, 14). One of the earliest studies using a metabolic chamber reported that accumulated fat oxidation over 24 h (24-h fat oxidation) was increased by exercise (4). However, it is likely that the subjects were in a state of negative energy balance on the day with exercise, which, per se, would have elevated fat oxidation (24). Negative energy balance over 24 h is associated with increased fat oxidation, i.e., the oxidized fuel mix contains more fat than the mixture supplied by the diet (11, 12, 21). Consensus in the literature states that if exercise is accompanied by increased energy intake to achieve an energy-balanced condition, 24-h fat oxidation remains similar to that of the sedentary day (22–25).

In addition to energy balance, 24-h fat oxidation may also be affected by energy flux, which refers to the absolute level of energy intake and expenditure under conditions of energy balance (2). In one study, subjects performed exercise at 50% of maximal oxygen uptake ($V_{O_{2max}}$) for 60 min or remained sedentary, and energy balance of the two experiments was matched by adjusting energy intake (5). Compared with low energy flux in a sedentary condition, accumulated fat oxidation during 9 h of indirect calorimetry was higher in a high-energy flux condition with exercise.

Findings of abovementioned studies can be interpreted to mean that when exercise is performed the nutritional state affects 24-h fat oxidation. Exercise has little effect on 24-h fat oxidation when performed after breakfast (22, 23, 24, 25), but that performed before breakfast increases fat oxidation during 9 h postexercise (5). Compared with exercise performed after breakfast, 24-h fat oxidation was higher when exercise was performed before breakfast in our previous study (35). The mechanism responsible for the increase in 24-h fat oxidation on a day with exercise, if any, remains to be elucidated. However, a link between 24-h fat oxidation and transient energy deficit has been suggested as follows. Even though energy intake and expenditure over 24 h was matched in both experimental conditions, time course of energy balance varied according to the time when exercise was performed. Exercise performed before breakfast, when diurnal variation in energy content of the body reaches its nadir, further decreases body energy, i.e., a transient but large decrease in body energy content (35). Among macronutrients stored in the body, pool size of carbohydrate is the smallest, and metabolic response to changes in carbohydrate storage is more sensitive than those in fat and protein (11, 12). Recent studies suggest an underlying mechanism, which translates depletion of glycogen in liver and skeletal muscle to a sustained increase in whole body fat oxidation (19, 31). Taken together, available evidence suggests that exercise performed in the postabsorptive state depletes glycogen storage, which in turn increases 24-h whole body fat oxidation.

The objective of the present study was to examine the relation between 24-h fat oxidation and exercise-induced transient deficit in energy and/or carbohydrate. To this end, 24-h indirect calorimetry was performed on three occasions with a session of 100 min of exercise before breakfast (AM), after

Address for reprint requests and other correspondence: K. Tokuyama, Doctoral Program in Sports Medicine, Graduate School of Comprehensive Human Sciences, Univ. of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki, Japan 305-8574 (e-mail: tokuyama@taiiku.tsukuba.ac.jp).

lunch (PM), or two sessions of 50 min of exercise before breakfast and after lunch (AM/PM). All experimental conditions were designed to be energy balanced and matched for energy flux over 24 h. Physiological significance of transient energy deficit affecting 24-h energy metabolism was discussed.

METHODS

Subject characteristics. Nine young male endurance athletes were recruited in this study after giving their written informed consent. This study was approved by the ethics committee of University of Tsukuba. Physical characteristics of subjects were 23.2 ± 2.7 years of age, 168.4 ± 5.7 cm of height, 59.5 ± 1.2 kg of body wt and 11.6 ± 0.6% of body fat. Their $\dot{V}O_{2max}$ was 71.7 ± 6.4 ml·kg^{-1}·min^{-1}. All subjects were free from pathological condition and none were taking any medications or supplements. One week before the 24-h calorimetry, subjects spent a night in a metabolic chamber to get acclimated to the measurement condition.

Prestudy evaluation. The velocity of treadmill running corresponding to 65% of the individual $\dot{V}O_{2max}$ was determined from an incremental exercise test on a motor-driven treadmill (ORK-7000, Ohtake Root Kogyo, Iwate, Japan) at least 1 wk before the main experiment. Initial velocity of the maximum running test was 200 m/min for 3 min, and it was increased by 20 m/min every 2 min until 280 m/min, then it was increased 10 m/min every 1 min until exhaustion. Oxygen uptake ($\dot{V}O_2$) was considered to be maximal when at least two of the following three criteria were met: *1*) $\dot{V}O_2$ reached plateau, *2*) heart rate exceeded 90% of the age-predicted maximal value, and *3*) respiratory exchange ratio increased above 1.10. The highest $\dot{V}O_2$ for a consecutive 30 s during the test was taken as $\dot{V}O_{2max}$ of the subject. Respiratory gas analysis was continuously performed on a breath-by-breath basis using the computerized standard open circuit technique (AE-310s Minato Medical Science, Osaka, Japan). Body composition was measured by bioimpedance method (BC-118E, Tanita, Tokyo, Japan).

Experimental protocol. The study was a randomized repeated measures design including three 24-h calorimetry measurements comprising AM, PM, and AM/PM exercise sessions. A washout period of at least 1 wk was inserted between each session. In each trial, subjects stayed in a metabolic chamber for 42 h beginning the day before exercise session (*day 1*, 22:00) until the day after exercise session (*day 3*, 16:00). During the daytime before entering the metabolic chamber, subjects were instructed to refrain from performing strenuous exercise and consumption of beverages containing energy, caffeine, or alcohol. In the metabolic chamber, subjects slept for 7 h from 23:00 to 6:00, and three meals (breakfast at 8:30, lunch at 12:30, and supper at 18:00) were provided. Subjects were instructed to remain awake and to keep sedentary position other than the prescribed exercise session and bedtime by the protocol.

On *day 2*, subjects performed a session of 100 min of exercise beginning at 6:30 (AM), at 16:00 (PM), or two sessions of 50 min of exercise at 6:30 and at 16:00 (AM/PM) at 65% of $\dot{V}O_{2max}$ using a treadmill (T1201, Johnson Health Tech Japan, Tokyo, Japan). In addition to 100 min of running, subjects were instructed to perform 15 min of warm-up activity twice a day (6:15 and 15:45). Subjects were allowed to leave the chamber for 30 min to take a shower (22:00–22:30). Energy metabolism during the 30-min break was estimated from the metabolic equivalent of taking shower as 2.0 metabolic equivalents and respiratory quotient (RQ) immediately before the break, as previously described (35). On *day 3*, subjects followed the same protocol of *day 2* except the exercise session, and exited the chamber at 16:00. Twenty-four-hour energy expenditure and nutrients oxidation calculated from 6:00 of *day 2* to 6:00 of *day 3* were compared among the three experimental conditions.

Experimental meals were designed to achieve individual energy balance, assuming resting metabolic rate to be 24.0 kcal·kg^{-1}·day^{-1} and physical activity factor to be 1.75 (2,464 ± 75 kcal/day) in *day 1*, 2.48 (3,544 ± 127 kcal/day) in *day 2*, and 1.75 in *day 3* (1,587 ± 47 kcal for breakfast and lunch), according to the estimated energy requirement for Japanese (26). Expressed as a percentage of total energy, the standardized meals consist of 15% protein, 25% fat and 60% carbohydrate. The contributions of breakfast, lunch, and supper to total 24-h energy intake were 32%, 34%, and 34%, respectively.

Measurement. Energy metabolism was measured with a room-size metabolic chamber (Fuji Medical Science, Chiba, Japan). The airtight chamber measures 2.00 × 3.45 × 2.10 m, having an internal volume of 14.49 m^3. The chamber is furnished with an adjustable hospital bed, desk, chair, and toilet. Air in the chamber was pumped out at a rate of 120 l/min. Temperature and relative humidity of incoming fresh air were controlled at 25.0 ± 0.5°C and 55.0 ± 3.0%, respectively. Concentrations of oxygen (O_2) and carbon dioxide (CO_2) in outgoing air were measured by an online process mass spectrometer (VG PrimδB, Thermo Electron, Winsford, UK). The mass spectrometry measurements, defined as the standard deviation for continuous measurement of calibration gas mixture (O_2: 15%; CO_2: 5%), were 0.0016 and 0.0011% for O_2 and CO_2, respectively. At every 5 min, $\dot{V}O_2$ and CO_2 production rate ($\dot{V}CO_2$) were calculated using an algorithm for improved transient response (37).

Macronutrient oxidation and energy expenditure were calculated from $\dot{V}O_2$, $\dot{V}CO_2$, and urinary nitrogen excretion (10). Rates of glucose, fat, and protein oxidation were computed from the following equations, assuming urinary nitrogen excretion rate (N) to be constant during the calorimetry: glucose oxidation (g/min) = 4.55 $\dot{V}CO_2$ (l/min) − 3.21 $\dot{V}O_2$ (l/min) − 2.87 N (g/min); fat oxidation (g/min) = 1.67 $\dot{V}O_2$ (l/min) − 1.67 $\dot{V}CO_2$ (l/min) − 1.92 N (g/min); and protein oxidation (g/min) = 6.25 N (g/min).

Subsequently, energy production by each nutrient was calculated by taking into account the caloric equivalent of the three substrates, i.e., 3.74 kcal/g glucose, 9.50 kcal/g fat, and 4.10 kcal/g protein. It is conceivable that tissue glycogen rather than glucose is the predominant form of carbohydrate being oxidized during exercise. An equation for complete oxidation of glycogen and its caloric equivalent are different from those of glucose. However, estimates of energy expenditure by carbohydrate oxidation in terms of energy expenditure in a unit time (kcal/min) are robust, regardless of whether glucose or glycogen is oxidized in the body (10, 32). Therefore, glucose oxidation was discussed as carbohydrate oxidation in the present study. Energy and nutrient balance relative to the beginning of 24-h calorimetry were estimated as the difference between the input and output. For example, relative energy balance was defined as a function of time (*t*) since 6:00 of *day 2*: relative energy balance (*t*) = accumulated energy intake (*t*) − accumulated energy expenditure (*t*).
This represents an "apparent" balance, because not all the dietary nutrients are absorbed during the short period (4, 34).

Physical activities including nonexercise activity were estimated using a wristwatchlike device, ActiGraph (Ambulatory Monitoring, NY) with zero crossing mode, as the number of times the activity signal crossed the zero reference point per minute (counts/min).

Statistical analysis. Data in the text and figures were given as means ± SE of the experimental condition. Statistical analyses were performed using SPSS statistical software (Version 20, IBM Japan, Tokyo, Japan). Mean values of the three trials were compared using one-way repeated measures ANOVA, with post hoc pair-wise comparisons using Bonferroni correction. Statistical significance was set at the $P < 0.05$ level.

RESULTS

All subjects completed the three trials, and there were no significant differences in body mass, body fat, and fat-free mass among the trials. During exercise, energy expenditure was similar, but oxidation of fat and carbohydrate were different among the trials ($P < 0.01$). Fat oxidation during exercise in descending order were exercise performed before breakfast

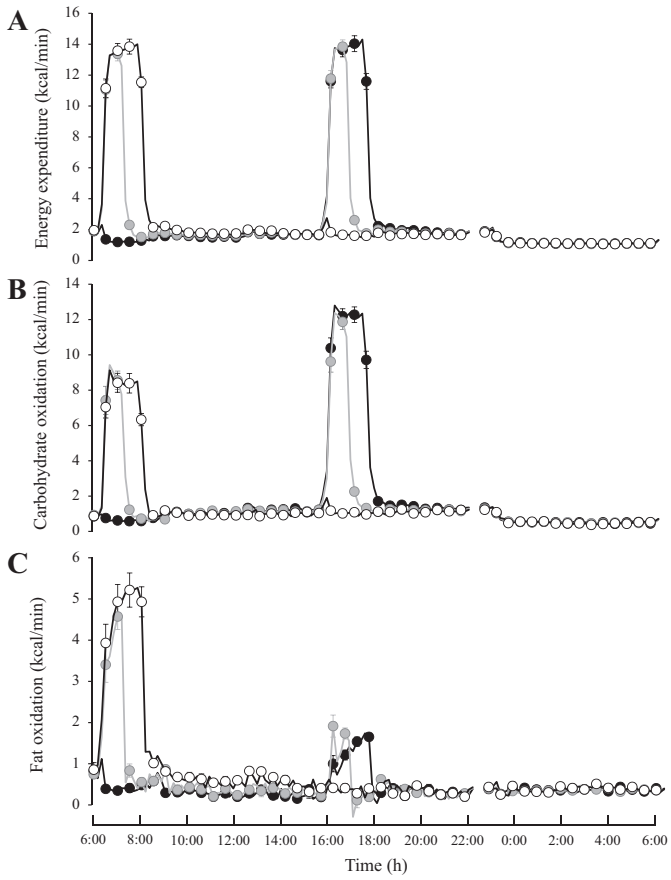

Fig. 1. Time course of energy expenditure and substrate oxidation. Energy expenditure (*A*), carbohydrate oxidation (*B*), and fat oxidation (*C*) were calculated as kcal/min. Plots are mean ± SE at every 30 min for trial with a session of 100 min of exercise before breakfast (open circles), after lunch (closed circles), and two sessions of 50 min of exercise before breakfast and after lunch (gray circles).

(AM: 481 ± 41), two split sessions before breakfast and after lunch (AM/PM: 279 ± 22), and after lunch (PM: 131 ± 5 kcal/100 min). Conversely, carbohydrate oxidation during exercise in descending order were PM (1,189 ± 45), AM/PM (992 ± 50), and AM (815 ± 52 kcal/100 min) (Fig. 1).

Accumulated 24-h energy expenditure was similar among the trials (AM: 3,540 ± 124; AM/PM: 3,525 ± 128; PM: 3,487 ± 120 kcal/24 h; $P = 0.15$), and it was balanced with energy intake, i.e., energy balance over 24 h was indistinguishable from 0 and similar among experimental conditions (AM: +4 ± 74; AM/PM: +19 ± 60; PM: +58 ± 60 kcal/24 h; $P = 0.15$). Accumulated 24-h fat oxidation in descending order were AM (1,142 ± 97), AM/PM (809 ± 88), and PM (608 ± 46 kcal/24 h), and that of AM was significantly higher than that of AM/PM and PM exercise condition ($P < 0.01$). Contrarily, accumulated 24-h carbohydrate oxidation in descending order were PM (2,558 ± 110), AM/PM (2,374 ± 114), and AM (2,062 ± 96 kcal/24 h) ($P < 0.01$). Urinary nitrogen excretion was not significantly different among the three trials (AM: 13.1 ± 1.2; AM/PM: 13.3 ± 1.5; PM: 12.5 ± 1.3 g/day; $P = 0.39$).

Although energy intake and expenditure over 24 h was matched in all experimental conditions, time course of relative energy balance was different during the first half of *day 2* (6:00–18:00, Fig. 2). Exercise performed before breakfast decreased relative energy balance to −1,460 ± 49 kcal, whereas the corresponding value for the trial with exercise performed after lunch was −219 ± 11 kcal. Twenty-four-hour averages of relative energy balance were positive and different among exercise conditions (AM: 100.1 ± 49.4; AM/PM: 347.7 ± 37.9; PM: 652.9 ± 42.3 kcal; $P < 0.01$). Nadir ($r = -0.72$, $P < 0.01$) and 24-h average ($r = -0.52$, $P < 0.01$) of relative energy balance were negatively correlated with 24-h fat oxidation (Fig. 3). Relative carbohydrate balance during the first half of *day 2* in descending order were PM, AM/PM, and AM, but it was in opposite order during the second half of *day 2*, i.e., AM, AM/PM, and PM in descending order. Nadir of relative carbohydrate balance was negatively correlated with 24-h fat oxidation ($r = -0.40$, $P < 0.05$), but its average over 24 h was not significantly correlated with 24-h fat oxidation ($r = 0.35$, $P = 0.07$). Time course of fat balance was different throughout 24-h indirect calorimetry, and values in descending order were PM, AM/PM, and AM.

The day after exercise session (*day 3*), subjects followed the same protocol from getting up at 6:00 until exiting the chamber at 16:00. During the 10 h of *day 3*, total energy expenditure (AM: 915 ± 55; AM/PM: 923 ± 47; PM: 910 ± 41 kcal/10 h;

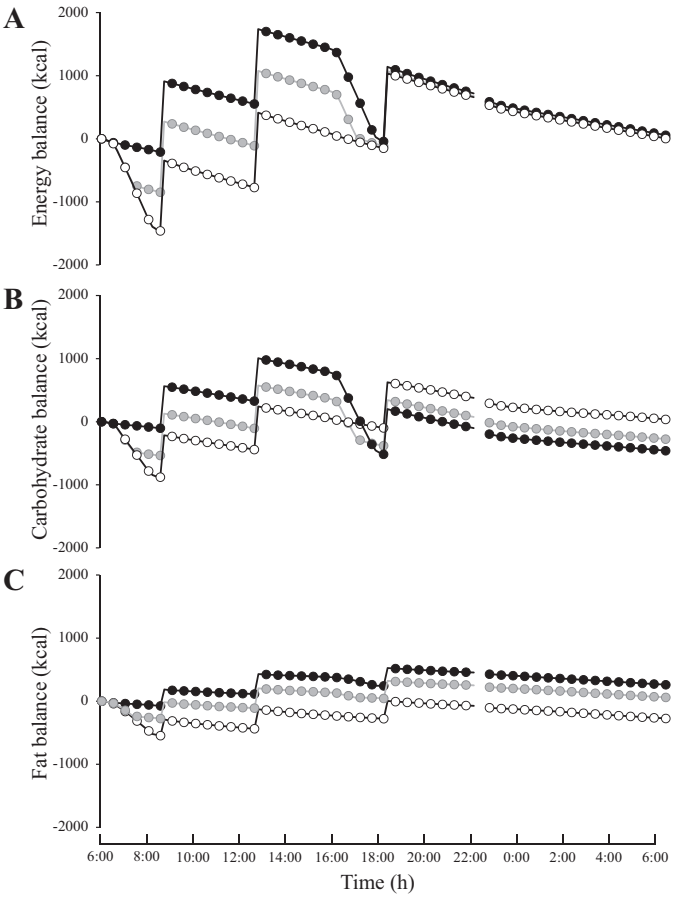

Fig. 2. Time course of relative energy balance and substrate balance. Energy (*A*), carbohydrate (*B*), and fat (*C*) balance were calculated by setting the initial reference value at 6:00 of *day 2*. Means for trial with a session of 100 min of exercise before breakfast (open circles), after lunch (closed circles), and two sessions of 50 min of exercise before breakfast and after lunch (gray circles) were plotted at every 30 min.

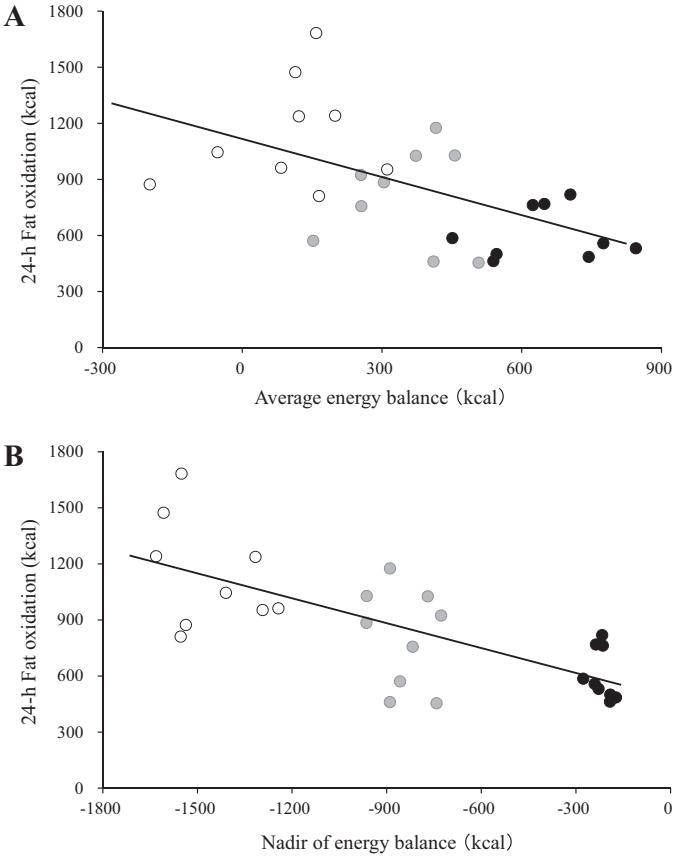

Fig. 3. Relation between relative energy balance and 24-h fat oxidation. Relations of 24-h fat oxidation to average ($r = -0.52$, $P < 0.01$) and nadir ($r = -0.72$, $P < 0.01$) of relative energy balance were plotted on *A* and *B*, respectively. All nine subjects completed three trials with different exercise protocol; a session of 100 min of exercise before breakfast (open circles), after lunch (closed circles), and two sessions of 50 min of exercise before breakfast and after lunch (gray circles).

$P > 0.7$), accumulated carbohydrate oxidation (AM: 565 ± 23; AM/PM: 557 ± 26; PM: 532 ± 216 kcal/10 h; $P = 0.09$), and accumulated fat oxidation (AM: 175 ± 21; AM/PM: 191 ± 20; PM: 216 ± 25 kcal/10 h; $P = 0.17$) were not significantly different among the three trials. Also, urinary nitrogen excretion during the 10 h was not significantly different among the three trials (AM: 6.9 ± 1.4; AM/PM: 6.9 ± 0.7; PM: 6.4 ± 0.4 g/10 h; $P > 0.7$).

There were no significant differences in overall mean heart rate (AM: 69 ± 2; AM/PM: 70 ± 2; PM: 69 ± 2 beats/min; $P > 0.4$) and in activity counts (AM: 83 ± 10; AM/PM: 73 ± 7; PM: 80 ± 8 counts/min; $P > 0.5$) during *day 2* among the three trials.

DISCUSSION

Fat oxidation during exercise is suppressed if carbohydrate is ingested before exercise (3, 7, 8, 9, 18, 27, 38), and the suppressive effect of carbohydrate ingestion on fat oxidation persists at least for 4 h after a meal (27). Consistent with the literature, fat oxidation during exercise was higher when exercise was performed in the postabsorptive state (AM) compared with that during exercise performed after lunch (PM). However, it was possible that the differences in substrate oxidation during exercise could be offset during the postexercise period as suggested in some experimental conditions (24, 25). The main finding of the present study is that 24-h fat oxidation was higher when exercise was performed before breakfast (AM) compared with exercise performed after lunch (PM), and the difference in 24-h fat oxidation between the two exercise protocols (1,142 ± 97 vs. 608 ± 46 kcal/24 h, +92%) was highly significant ($P < 0.01$). When exercise was split into two sessions, before breakfast and after lunch (AM/PM), accumulated fat oxidation was a value between 24-h fat oxidation of the other experimental conditions (809 ± 88 kcal/24 h). In our previous study, difference in 24-h fat oxidation was modest between exercising conditions (+18%): exercise for 60 min at 50% of $\dot{V}O_{2max}$ performed before breakfast and that performed after breakfast (35). Increased exercise volume and/or wider difference in exercise timing in the present study may have highlighted the effect of exercise performed before breakfast on 24-h fat oxidation.

Time course of energy expenditure revealed a prolonged increase in energy expenditure after a bout of exercise. During 3 h from 9:00 to 12:00, energy expenditure in AM trial, i.e., postexercise period (330 ± 15), was higher than that of PM, i.e., resting metabolic rate (280 ± 12 kcal/3 h, $P < 0.01$). Similarly, energy expenditure from 18:30 to 21:30 was higher in PM trial (342 ± 13) than that of AM (307 ± 13 kcal/3 h, $P < 0.01$). However, energy expenditure during sleep (AM 471 ± 18 vs. PM 470 ± 18 kcal/7 h, $P > 0.9$) and the day after the exercise session (915 ± 55 vs. 910 ± 41 kcal/10 h, $P > 0.7$) were indistinguishable between experimental conditions, suggesting that there was little residual effect of exercise on energy expenditure.

Exercise performed before breakfast induced transient energy deficit, and it was related to 24-h fat oxidation. Nadir and average of relative energy balance was negatively correlated with 24-h fat oxidation. This finding was strengthened by correlation analysis of pooled data of our previous (35) and present study. Since volume of exercise and energy flux were

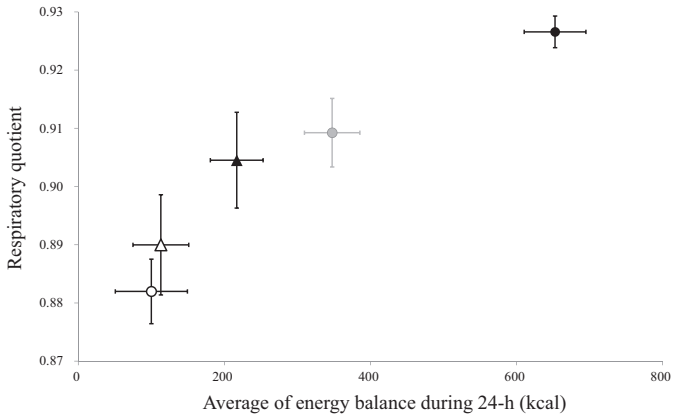

Fig. 4. Relation between relative energy balance and respiratory quotient in combined data pool. All nine subjects in the present study completed three trials with different exercise protocol; session of 100 min of exercise before breakfast (open circle), after lunch (closed circle), and two sessions of 50 min of exercise before breakfast and after lunch (gray circle). In our previous study (35), 12 men completed two trials with different exercise protocol; session of 60 min exercise at 50% of $\dot{V}O_{2max}$ before breakfast (open triangle) and after breakfast (closed triangle). Correlation coefficient between 24-h average of relative energy balance and respiratory quotient was 0.61 ($P < 0.01$).

different between the two studies, 24-h RQ was used as index of 24-h fat oxidation. Combined data clearly showed that lower average of relative energy balance was related to lower RQ ($r = 0.61$, $P < 0.01$) (Fig. 4). Hence, the transient energy deficit was implied as a factor, which increases 24-h fat oxidation, even when energy intake and expenditure over 24 h is matched.

The nadir of relative carbohydrate balance in descending order was PM (-517 ± 39 kcal), AM/PM (-536 ± 34 kcal), and AM (-879 ± 56 kcal) exercise condition, and the difference was statistically significant ($P < 0.01$). The transient decrease in relative carbohydrate balance in the AM exercising trial was roughly equivalent to 38% of the whole body glycogen storage, assuming that whole body glycogen store in a postabsorptive state is ~2,300 kcal (16). Consistently, a study using magnetic resonance spectroscopy reported that exercise (at 70% of $\dot{V}O_{2max}$ for 83 min) performed before breakfast depleted liver and skeletal muscle glycogen by 51 and 55%, respectively (6). Modest but significant negative correlation between nadir of relative carbohydrate balance and 24-h fat oxidation ($r = -0.40$, $P < 0.05$) suggests that differences in exercise-induced glycogen depletion affects accumulated fat oxidation over 24 h.

Cellular energy deficit is sensed by AMP-activated protein kinase (AMPK)(15). When activated, AMPK increases fatty acid oxidation in skeletal muscle (38). AMPK activation by exercise is higher in the fasted state compared with fed state (9), and recent studies provided a potential molecular link between glycogen depletion and AMPK activation (31). Since exercise-induced AMPK activation persists during the postexercise period for at least 150 min (36), it is plausible that transient energy deficit after exercise performed in a postabsorptive state activates AMPK to increase 24-h fat oxidation.

Although the present study did not address the following issues, fat oxidation is influenced by circulating substrates and hormones, such as glucose (1) and insulin (39). Moreover, higher plasma FFA level in a postabsorptive state favors fat oxidation by increasing substrate availability. Furthermore, a state of negative energy balance such as prolong fasting (29) and exercise (28) increases the unsaturated/saturated ratio of plasma FFA, which also stimulates fat oxidation (33). The role of these circulating factors to mediate interaction between exercise and nutritional status affecting 24-h fat oxidation remains to be studied.

To evaluate the translational potential of the present study, some considerations are required. The present study compared the effect of a single bout of exercise performed at different times of day on 24-h fat oxidation with energy-balanced condition, and findings of the present study cannot be extrapolated to the chronic effects of the postabsorptive exercise to reduce body fat. Because carbohydrate storage capacity of the body is limited, if exercise performed before breakfast oxidizes more fat and saves carbohydrate in energy-balanced condition, positive carbohydrate balance would eventually be counterbalanced by increase in its oxidation. Alternatively, in free-living conditions, the expanded glycogen storage may inhibit subsequent energy intake, because carbohydrate balance is a strong predictor of subsequent ad libitum food intake (13, 30). Finally, in the present study, subjects ran 21.0 ± 0.5 km (13.0 ± 0.3 miles), which was the reason young male athletes were recruited. To generalize the present results, studies with subjects of limited capacity to perform endurance exercise, such as middle-aged obese subjects, are required. Furthermore, sex difference in fat oxidation during and after exercise (17) warrants studies with women.

In conclusion, even in energy-balanced condition, 24-h fat oxidation is increased if exercise-induced transient energy deficit is significant, which is likely to be observed during exercise performed before breakfast.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

K.I., R. Kawabuchi, I.P., R. Kurihara, K.Y., Y.N., and K.T. conception and design of research; K.I., R. Kawabuchi, I.P., and R. Kurihara performed experiments; K.I. prepared figures; K.I. and K.T. drafted manuscript; M.K., M.H., S.O., and H.O. analyzed data; K.Y., Y.N., and K.T. edited and revised manuscript; K.T. approved final version of manuscript.

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