An arginine, alanine, and phenylalanine mixture increases synthesis of ketone bodies during low-intensity exercise via stimulating glucagon secretion in men with obesity

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Abstract During exercise, levels of several hormones are acutely increased in the blood. We previously reported that pre-exercise ingestion of a specific combination of amino acids (arginine, alanine, and phenylalanine; A-mix) increases fat mobilization and ketone body synthesis by increasing secretion of adrenalin and glucagon in healthy active young men. Herein, we sought to determine whether this acute hormone response could be induced upon administration of A-mix combined with exercise in patients with obesity during periods of low-intensity exercise. We performed a randomized crossover study of eleven middle-aged men with obesity without regular exercise habits, administered either A-mix (3 g/dose) or a placebo (3 g of dextrin/dose). Thirty minutes after ingestion, each subject subsequently performed workload tests on a cycle ergometer at 40% of peak oxygen consumption for 1 h. Following oral intake of A-mix, the concentration of plasma ketone bodies was significantly increased during exercise. This was accompanied by a significant increase in the area under the concentration-time curve for glucagon. Taken together, these results indicate that pre-exercise ingestion of the A-mix supplement significantly accelerated hepatic ketone body synthesis via stimulation of glucagon secretion during exercise in men with obesity.

Keywords: pre-exercise nutrition, amino acid supplementation, metabolism, hormones

Introduction

Obesity is a worldwide public health problem1, and a major risk factor for insulin resistance, type 2 diabetes, atherosclerosis, stroke, hypertension, and some types of cancer2. Obesity results from a chronic imbalance in energy metabolism. To prevent and/or mitigate these concerns, weight loss through dietary restriction and regular exercise is considered the best available non-surgical, non-pharmacologic treatment strategy3).

Regular exercise is one important element in the approach to prevent obesity4,5. According to the exercise prescription recommended in the American College of Sports Medicine guidelines, 45-60 min of exercise should be targeted to ensure sufficient energy expenditure in obese people. In particular, the blood levels of several hormones such as catecholamines, glucagon, growth hormone and cortisol are increased by acute exercise6,7. Moreover, several studies reported on acute exercise and lipid utilization, and related hormones in obese subjects8,9. Therefore, we hypothesize that a combination of acute exercise and the factors that affect the secretion of some of these hormones may be effective for increasing fat catabolism.

We recently focused our research efforts on amino acids (AAs) that affect glucagon secretion, as it was previously reported that ingestion or infusion of some AAs increases insulin and/or glucagon concentrations in the blood10. Glucagon is one of the key hormones implicated in fat catabolism during exercise11,12. Tan et al. reported that glucagon infusion acutely increased energy expenditure in humans13. Moreover, glucagon administration in rodents was shown to increase brown adipose tissue mass and activity14. We hypothesized that a specific combination of AAs that affects glucagon secretion may acutely promote fat catabolism and energy expenditure when combined with exercise. Several reports suggested that when combining AAs with similar functions, the additive effect is expected for the mixture15,16. Moreover, arginine and phenylalanine increase GTP cyclohydrolase-I expression and activity that increases tetrahydrobiopterin availability for NO synthesis and aromatic AA hydroxylation17. Therefore, in earlier reports, we developed a new AA mixture containing arginine, alanine, and phenylalanine (A-mix), and investigated the acute effects of A-mix supplementation combined with 60-min moderate-intensity (50% of maximal oxygen uptake [VO2max]) exercise using cycle ergometry. Pre-exercise ingestion of A-mix increased levels of ketone bodies and glycerol in the blood both during...
and after a bout of exercise in healthy active young men, by increasing secretion of adrenalin and glucagon\(^{18}\). Herein, we sought to determine whether this acute response induced by administration of A-mix combined with exercise also occurs in patients with obesity during periods of low-intensity exercise. Previous cross-sectional studies reported that the exercise intensity that corresponds to a maximal fat oxidation is 42% of \(\text{VO}_2\text{max}\)\(^{19}\). Therefore, we conducted a randomized, double-blind, placebo-controlled crossover study to investigate glucagon secretion induced by A-mix and fat catabolism combined with 40% of \(\text{VO}_2\text{peak}\) intensity exercise in middle-aged men with obesity without regular exercise habits.

**Materials and Methods**

**Trial design.** This was a randomized, double-blind, placebo-controlled crossover study, conducted at the Chiyoda Paramedical Care Clinic in Chiyoda-ku, Japan, from June 2015 to August 2015. The study protocol was approved by the Institutional Review Board of the Chiyoda Paramedical Care Clinic (No.15061803) and the Meiji Institutional Review Board (No.57). All study participants provided written informed consent prior to participation in the study. The study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. The study protocol was registered in the UMIN Clinical Trials Registry (UMIN000018161) on July 1, 2015.

**Participants.** Participants were recruited via a contract research organization (CRO). Overweight (BMI \(\geq 25\) but \(< 30\) kg/m\(^2\)) men aged 30 to 50 years old without regular exercise habits were included in the study. A regular exercise habit was defined as engaging in at least 30 min of exercise twice a week for more than one year, according to the criteria of the National Health and Nutrition Examination Survey\(^{20}\). Exclusion criteria consisted of individuals with a history or current condition of severe disease (such as liver disorder, cardiovascular disorder, respiratory disorder, endocrine disorder, and/or metabolic disorder), severe systemic disease, heavy drinkers (\(\geq 40\) g of ethanol per day), smokers, those with extremely irregular eating patterns due to shift work or late-night work, those who regularly consumed functional foods or nutritional supplements, those who donated blood over the past 6 months, patients with phenylketonuria or hyperphenylalaninemia, and those who were judged ineligible by the study physician due to abnormal blood and urine tests or for other reasons. Characteristics of the study participants are included in Table 1.

**Experimental procedures.** Study participants visited the clinic a total of three times throughout the study. During the first visit, all participants underwent a physical examination, and a baseline blood sample (10 mL) was collected for analysis of the following parameters: platelet, white blood cell and red blood cell counts, levels of hemoglobin, hematocrit, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, gamma-glutamyl transferase, total bilirubin, albumin, total protein, blood urea nitrogen, creatinine, uric acid, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glucose, sodium, potassium, and chloride. Body weight and percent fat mass were measured using multi-frequency bioelectrical impedance analysis (Lookin’Body 3.2, Biospace, Seoul, Korea). Height was measured to the nearest 0.1 cm using a portable stadiometer (AD-6351, A&D Co., Ltd., Tokyo, Japan) at baseline to estimate BMI. BMI was computed as weight in kilograms divided by squared height in meters. The physical working capacity (PWC) at 75% of the maximal heart rate (HR) was measured on a ramp test (8 W/min) using a bicycle ergometer (Aero Bike 75XL-III, Konami Sports Life Co., Ltd., Kanagawa, Japan). The age-related maximal HR was calculated as: \(209 - 0.69 \times \text{age} \), in beats per min (BPM). Results of the PWC75%\(\times \text{HRmax/weight} \) were used to estimate the power output equivalent to 40% of \(\text{VO}_2\text{peak}\)\(^{21}\).

The remaining two study visits were separated by at least six days. On the day of the second and third visits, the subjects consumed meals provided at least 6 h before each test. All meals had the same carbohydrate:fat:protein ratio (71:16:13) and contained 612 kcal. The subjects consumed no food or drink except water from the last meal to the start of each test. Individual tests were performed at a similar time of day for each subject (\(\pm 3\) h) to avoid any influence of circadian rhythm on the results. Each subject continued his usual diet and was instructed to refrain from binge eating, strenuous exercise throughout the study, and drinking alcohol for 24 hours prior to each test. Dietary intake was self-recorded by the subjects from their first visit until their last.

During the second and third visits, subjects participated in the main study tests. After measurement of blood pressure and HR, blood samples were drawn from the antecubital vein. The subjects were randomized to ingest 50 mL

<table>
<thead>
<tr>
<th>Table 1. Characteristics of subjects</th>
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<tbody>
<tr>
<td>Age (years)</td>
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<tr>
<td>Height (cm)</td>
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<tr>
<td>Body weight (kg)</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
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<tr>
<td>Percent fat mass (%)</td>
</tr>
<tr>
<td>(\text{VO}_2\text{ max} ) (ml/kg/min)</td>
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</table>

Values are mean ± SD (n =11)
of ordinary tap water and a cellulose capsule containing either 3 g of A-mix (Kyowa Hakko Bio Co., Ltd., Tokyo, Japan) as the active sample or 3 g of dextrin (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan) as the placebo (designated 0 min). The treatments were subsequently switched during the crossover phase of the study. After sitting for 30 min (rest period), the subjects mounted a cycle ergometer and commenced cycling for 60 min at a constant power output equivalent to 40% of V\textsuperscript{\textsubscript{O2peak}} (exercise period). In order to prevent dehydration, 200 ml of tap water was consumed by all subjects at 30 min during the exercise. After exercising, subjects rested for 60 min in the supine position (post-exercise period). Blood samples were collected every 30 min throughout the test. HR was tracked from the earlobe during exercise. Blood pressure and HR were recorded again at the end of the post-exercise period. The tests were conducted in a quiet controlled room environment at a temperature of 21 ± 2°C and humidity of 45 ± 5%. The study design is summarized in Fig. 1.

**Blood sampling.** The assays used to measure glucagon, insulin, blood glucose, acetoacetic acid, 3-hydroxybutyrate, FFAs, growth hormone, adrenalin, and blood chemistry panels were performed at the LSI Medience Corporation (Tokyo, Japan), while the glycerol assay was performed at IMUH Co., Ltd. (Tokyo, Japan) as described previously\textsuperscript{18}). In brief, glucagon concentrations were measured by a double-antibody radioimmunoassay (Glucagon RIA SML, Euro-Diagnostica AB, Malmö, Sweden), while insulin levels were measured by a chemiluminescent immunooassay (Architect Insulin, Abbott Japan, Japan). Enzymatic methods were used to measure the levels of blood glucose (Iatoro LQ GLU, Unitica, Japan), acetoacetic acid, 3-hydroxybutyrate (Total Ketone Bodies, Kainos, 3-HB Kainos, Kainos Co., Ltd., Japan), glycerol (Glycerol Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, MI, USA), and lactate (Detamina-LA, Kyowa Medex Co., Ltd., Japan). Blood FFAs (NEFA-SS Eiken, Eiken Chemical Co., Ltd., Japan) were measured by the enzyme-ultraviolet (UV) method, and growth hormone (Access hGH, Beckman Coulter, Inc., USA) and cortisol (Access cortisol, Beckman Coulter, Inc., USA) concentrations were measured by a chemiluminescent enzyme immunoassay. Adrenalin and noradrenalin were measured using high-performance liquid chromatography (HLC-725CATT, Tosoh Corporation, Tokyo, Japan).

**Statistical analysis.** Data were expressed as mean ± standard deviation (SD), and were analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). The total area under the concentration-time curve (AUC) for each circulating plasma hormone was calculated using the Trapezium Rule. Repeated measures 2-factor analysis of variance (ANOVA, time-treatment) was used to examine differences between the biochemical parameters in the two tests. When the ANOVA showed significant effects or interactions between factors, Tukey’s post-hoc test was used to detect significant differences between the two treatments. To specifically compare the AUCs for each hormone between treatments, the experiment was divided into three time-phases (i.e., rest, exercise, and post-exercise). All variables were tested for normal distribution by the F-test using StatView-J 5.0 software (Abacus Concepts, Berkeley, CA, USA). Statistical significance of differences between the two experimental tests was analyzed by a paired-sample \textit{t} test using Microsoft Excel for normally distributed data and the Wilcoxon signed-rank test using the StatView-J 5.0 software for data with a skewed distribution. Correlations were tested using a two-
tailed Pearson’s test. Statistical significance was set at $P < 0.05$. To investigate the amount of the difference between the groups, effect size (Cohen’s d) was calculated.

Results

Cardiorespiratory responses. Two-factor ANOVA revealed significant effects of time post ingestion on the mean HR during exercise, but no significant treatment-time interaction. There were no differences in HR during exercise in subjects receiving either A-mix supplementation or a placebo (112 ± 10 vs. 108 ± 7 BPM, respectively, $P = 0.16$).

Biochemical parameters. Various biochemical parameters measured following ingestion of each of the two treatments are summarized in Fig. 2, and the AUCs during rest, exercise, and recovery are summarized in Table 2. Two-factor ANOVA showed significant effects of time post ingestion on the AUC, but no significant treatment-time interaction. However, the AUC for ketone bodies during exercise was significantly higher following supplementation with A-mix compared with the placebo, while the AUC for blood glycerol, FFAs, glucose, and lactate measurements did not differ between the two treatments.

Circulating hormones. The concentrations of circulating hormones following ingestion of A-mix and a placebo are summarized in Fig. 3, while the AUC for each during rest, exercise, and recovery is summarized in Table 3. Two-factor ANOVA showed a significant treatment-time interaction for plasma glucagon concentration (treatment, $P = 0.60$; time, $P < 0.01$; interaction, $P = 0.018$), with Tukey’s post-hoc test revealing significant differences between treatments at 30 min post-ingestion ($P < 0.05$). Moreover, the AUC for glucagon during exercise was significantly higher following supplementation with A-mix compared to the placebo ($P = 0.026$). Moreover, the [glucagon]:[insulin] ratio during exercise positively correlated with ketone bodies AUC ($r = 0.44$, $P = 0.038$, Fig. 4.). Two-factor ANOVA revealed significant effects of time post ingestion on blood levels of insulin, adrenalin, noradrenalin, growth hormone, and cortisol concentrations; but no significant treatment-time interaction, and no significant differences between the two treatments throughout the experimental period.

Discussion

This study investigated the acute effects of A-mix supplementation combined with low-intensity exercise in middle-aged men with obesity without regular exercise habits. The study showed that ingestion of the A-mix supplement significantly increased the concentration of ketone bodies during exercise, compared to ingestion of a placebo (Table 2). The amount of fat oxidation from adipose tissue can be estimated from the production of ketone bodies, since acetyl-CoA produced from the oxidation of FFAs gives rise to ketone bodies via a reaction during the HMG-CoA cycle in the liver. These results suggest that the ingestion of A-mix stimulates hepatic ketone body synthesis, and that such an effect might lead, at least in part, to an increase in fat oxidation during exercise. Moreover, the ingestion of A-mix significantly increased the concentration of glucagon in the blood (Table 3), and the [glucagon]:[insulin] ratio positively correlated the AUC of ketone bodies during exercise. Therefore, the acceleration of ketone body synthesis by A-mix ingestion can be estimated via the increase in glucagon secretion. These findings lay the groundwork for further investigations on specific amino acid supplementation combined with exercise and related hormones in patients with obesity.

In this study, following A-mix ingestion, ketone levels during exercise were significantly increased compared to pre-exercise ingestion of the placebo, and the [glucagon]:[insulin] ratio during exercise was positively correlated with ketone bodies AUC (Fig. 4). One possible mechanism for the increase in ketone levels is via a glucagon-stimulated cascade. A-mix also significantly increased the concentration of glucagon during exercise. McGarry & Foster previously reported that elevation of the [glucagon]:[insulin] ratio suppresses the synthesis of malonyl-CoA and lipogenesis, with concomitant activation of fatty acid oxidation and ketogenesis. Simultaneous increases in hepatic carnitine and fatty acyl-CoA content further enhance β-oxidation and the production of ketone bodies such as acetoacetic acid and 3-hydroxybutyric acid. Therefore, increasing the [glucagon]:[insulin] ratio is the key to controlling hepatic fatty acid oxidation and production of ketone bodies.

The balance of several hormones, including glucagon, insulin, catecholamine, growth hormone, and cortisol, plays a critical role in fat catabolism, both at rest and during exercise. We speculate that elevated blood amino acid levels by A-mix ingestion directly leads to elevated glucagon secretion. This notion is supported by a previous study showing that specific amino acids stimulate insulin and glucagon secretion in humans. In a previous report, we suggested that ingestion of A-mix caused a significant increase in the concentration of catecholamines, especially adrenalin, both during and after exercise. In this study, however, no significant differences in levels of adrenalin and noradrenalin were noted between the different treatments. Blood adrenalin levels were previously found to increase with exercise intensity. Taken together, these studies clearly indicate that the primary effect of A-mix ingestion is in stimulating glucagon secretion, and that, depending on the exercise intensity, can contribute to glycemic control.

Our study has several noteworthy strengths. First, this trial adopted the research design of a placebo-controlled
Fig. 2 Concentrations of biochemical parameters assessed during the experimental trials: glycerol (A), FFAs (B), total ketone bodies (C), glucose (D), and lactate (E). Values are M ± SD (n = 11). A = A-mix treatment; FFAs = free fatty acids; P = placebo treatment.
Table 2. Effects of amino acid mixture on the AUC for biochemical parameters

<table>
<thead>
<tr>
<th>Rest (0-30 min)</th>
<th>Exercise (&gt;30-90 min)</th>
<th>Recovery (&gt;90-150 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-mix</td>
<td>Placebo</td>
</tr>
<tr>
<td>Glycerol (min·mg/L)</td>
<td>$97 \pm 18$</td>
<td>$94 \pm 33$</td>
</tr>
<tr>
<td>FFAs (min·mEq/L)</td>
<td>$14.0 \pm 4.1$</td>
<td>$12.2 \pm 3.8$</td>
</tr>
<tr>
<td>Total ketone bodies</td>
<td>$3.83 \pm 3.26$</td>
<td>$2.26 \pm 2.12$</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n =11). FFA = free fatty acids.

* $p$ value for the paired-sample $t$ test if data were normally distributed and the Wilcoxon signed-rank test if not.

Table 3. Effects of amino acid mixture on the AUC for circulating hormones

<table>
<thead>
<tr>
<th>Rest (0-30 min)</th>
<th>Exercise (&gt;30-90 min)</th>
<th>Recovery (&gt;90-150 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-mix</td>
<td>Placebo</td>
</tr>
<tr>
<td>Glucagon (min·g/L)</td>
<td>$4.59 \pm 1.39$</td>
<td>$4.14 \pm 1.66$</td>
</tr>
<tr>
<td>Insulin (min·mU/L)</td>
<td>$168 \pm 81$</td>
<td>$160 \pm 93$</td>
</tr>
<tr>
<td>Adrenalin (min·g/L)</td>
<td>$0.71 \pm 0.29$</td>
<td>$0.72 \pm 0.42$</td>
</tr>
<tr>
<td>Noradrenalin (min·g/L)</td>
<td>$8.17 \pm 1.19$</td>
<td>$8.58 \pm 2.42$</td>
</tr>
<tr>
<td>Growth hormone (min·µg/L)</td>
<td>$13.7 \pm 21.7$</td>
<td>$7.31 \pm 6.77$</td>
</tr>
<tr>
<td>Cortisol (min·mg/L)</td>
<td>$2.42 \pm 0.60$</td>
<td>$2.10 \pm 0.37$</td>
</tr>
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</table>

Values are mean ± SD (n =11).

* $p$ value for the paired-sample $t$ test if data were normally distributed and the Wilcoxon signed-rank test if not.
Fig. 3  Concentrations of circulating hormones during the experimental trials: glucagon (A), insulin (B), adrenalin (C), noradrenalin (D), growth hormone (E), and cortisol (F). Values are M ± SD (n = 11). A = A-mix treatment; P = placebo treatment.
double-blind cross-over randomized trial. Therefore, the findings are highly reliable. Second, in this study, we employed low-intensity exercise (i.e., 40% of VO2peak). Low- to moderate-intensity exercise may be better than high-intensity exercise for promoting loss of fat mass, because the substrate oxidation achieved during high-intensity exercise is primarily reflective of carbohydrate oxidation. In addition, it is wise to encourage moderate-intensity physical activity in patients with obesity, as it is more feasible and acceptable than high-intensity exercise. Therefore, supplements that promote fat mobilization during periods of low-intensity exercise are considered optimal for exercise motivation in patients with obesity.

In contrast, we must note some limitations. First, we did not investigate the oxygen uptake and whole body energy expenditure. Therefore, the data robustness may not be high. Second, this study only investigated 11 middle-aged men. Future clinical trials are necessary in humans with obesity, especially including women.

In conclusion, pre-exercise ingestion of the A-mix supplement significantly accelerated secretion of glucagon during exercise in men with obesity. Furthermore, fat catabolism - especially hepatic ketogenesis - during exercise increased significantly, indicating a shift towards fat catabolism. Thus, these results suggest that ingestion of A-mix stimulates hepatic ketone body synthesis, and this effect might lead to, at least in part, an increase in fat oxidation during exercise. Further studies are needed to investigate whether this acute response induced by the administration of A-mix is sustained if the supplement is ingested over several weeks.

**Conflict of Interests**

This work was supported by a grant from Meiji Co., Ltd. K Ueda, C Sanbongi, and S Ikegami are employees of Meiji Co., Ltd.

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**References**


8) Miyashita M. 2008. Effects of continuous versus accumulat-


