The impact of exhaustive exercise on metabolic profiles in young men: a metabolomics approach

Nobuhiko Akazawa (✉ nobuhiko.akazawa@jpnsport.go.jp)

Japan Institute of Sports Sciences

Mariko Nakamura

Japan Institute of Sports Sciences

Mana Otomo

Japan Institute of Sports Sciences

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Abstract

Background: Exercise-induced fatigue leads to reduction in the ability exert physical performance. Prolonged and intensive exercise stimulates several metabolic pathways to produce energy. The purpose of the present study was to investigate the impact of exhaustive exercise on metabolomic pathways.

Methods: Nine young recreationally active men were recruited to this study. Participants performed step incremental maximal exercise until maximum exhaustion. Saliva samples were collected pre- and post-exercise (immediately after exercise cessation) using a salimetric oral swab, and salivary metabolites were analyzed using capillary electrophoresis and time-of-flight mass spectrometry.

Results: Two hundred ten metabolites were detected, representing different clustering principle component structures between pre- and post-exercise. Orthogonal partial least squares discriminant analysis identified 29 metabolites with highly related variable importance for projection score and 16 metabolites significantly increased after exercise. Furthermore, increase in cyclohexylamine was positively correlated with an increase in fatigue on a visual analog scale.

Conclusion: The present study demonstrated that exhaustive exercise changed the saliva metabolomic pathways related to energy production including glycolysis, lipolysis, amino acid metabolism, amines, and ketone bodies.

Introduction

Prolonged and strenuous exercise causes fatigue, which reduces force and power and then obstructs ability to continue physical performance. Multiple theories have been put forward to explain the complex and interactive nature of fatigue.^[1] Fatigue causes internal environment changes that include a lack of oxygen and energy from glucose and fatty acids, an accumulation of carbon dioxide, hydrogen ions, lactate, and ammonia, and a heat loading.² In high-intensity exercise conditions (e.g., after onset of blood lactate accumulation (OBLA)), homeostasis of the internal environment is disrupted mainly by lactate, ammonia, and organic acid, which reduce the exercise workload or stop it completely.^[2] It is plausible that maintaining metabolism of nutrients such as carbohydrate, fatty acid, and amino acid is important for reducing exhaustive fatigue.

The human metabolism could be analyzed by the metabolome, which can identify and quantify the specific metabolites through a comprehension of biological compounds at the small molecule level.^[3,4] The analysis of nontargeting approaches also provides a new insight into the biophysiological mechanism of response to disease or exercise.^[5] Previous studies have demonstrated that fatigue symptom during competitive or intensive exercise were associated with changes in glucose and tricarboxylic acid (TCA) cycle metabolic profile.^[6] In addition, Monaf et al. (2018)^[7] found changes in fatty acids, neurotransmitters, and indole metabolism following prolonged maximal exhaustive exercise at moderate intensity. However, little is known regarding the relationship between the human metabolome

and exhaustive exercise-induced fatigue relatively short duration at high intensity. Therefore, the aim of the present study was to investigate the impact of exhaustive maximal exercise on fatigue and the metabolome. We performed a comprehensive comparison of saliva metabolites before and after exhaustion induced by gradually increased exercise.

Methods

Subjects

A total of 9 healthy and recreationally active young men participated in this study. All subjects in this study were medication-free and had no history of metabolic abnormalities such as diabetes mellitus, hypercholesterolemia, hypertension, and inflammatory disease. The present study was approved by the institutional review board at the Japan Institute of Sports Sciences. All subjects gave written informed consent prior to participating in the study. All study procedures were performed in accordance with relevant guidelines/regulations.

Study protocol

This study was a single-arm trial consisting of exhaustive cycling exercise using a metabolomics approach. The participants performed the exhaustive exercise test at least 3 h postprandially and had not consumed any caffeine and alcohol for 12 h prior to the exercise test. They completed a 5-min warm-up at 50-W, and then power output increased by 50 W every 8 min until reaching an exhaustive state modified on the basis of previous study.^[8] The state of exhaustive fatigue is defined as the time point where participant cannot maintain cadence, and the heart rate reaches 90% of the maximum heart rate. Heart rate was monitored using short-range radio telemetry (RS 800, Polar, Finland). We measured the average of oxygen uptake every 30 s during exercise test using online computer-assisted circuit spirometer (AE300S; Minato Medical Science, Osaka, Japan). Subjects were asked to assess fatigue using a visual analog scale (VAS) pre- and post- exercise.

Salivary metabolome

Saliva samples were collected using an oral swab cotton swab and a storage tube (Salimetrics oral swab; Salimetrics, USA) before and immediately after the exhaustive exercise. Participants were asked to abstain from any food and drink before the saliva collection. Before and after exercise, they sat and rinsed their mouth with distilled water 3 times, then rested for at least 5 min, both of pre- and post-exercise. Saliva production was stimulated by chewing on cotton for 1 min at a rate of 1 chew/s.^[9] The obtained saliva samples were separated from the cotton by centrifugation at 1500 g, and the samples were frozen at -80 °C until analysis.

Metabolite levels were determined using capillary electrophoresis and time-of-flight mass spectrometry (CE-TOFMS) analysis. A saliva sample of 25 µL and Mili-Q water of 25 µL were combined with 400 µmol/L of commercial standard solution (H3304-1002; Human Metabolome Technologies, Japan) and passed through a 5 kDa cut-off filter to remove proteins and macromolecules. The filtrate was

analysed using CE-TOFMS performed using an Agilent capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) as described previously.^[10] Briefly, cationic metabolites were analyzed through a fused silica capillary (50 µm internal diameter, 80 cm length) with a commercial buffer as the electrolyte. The sample was injected at a pressure of 5 mbar for 10 s. The applied voltage was set at 28 kV. Electrospray ionization-mass spectrometry was conducted in the positive ion mode. The spectrometer was scanned with mass-to-charge ratio (m/z) ranging from 50 to 1000. Anionic metabolites were analyzed through a fused silica capillary (50 µm internal diameter, 80 cm length) with a commercial buffer as the electrolyte. The sample was injected at a pressure of 50 mbar for 25 s. The applied voltage was set at 30 kV. Electrospray ionization-mass spectrometry was conducted in the negative ion mode. The spectrometer was scanned using m/z ratios ranging from 50 to 1000. The obtained data were analyzed using proprietary autonomic integration software (MasterHabs; Human Metabolome Technologies, Tsuruoka, Japan). Each metabolite was identified and quantified by the peak information including m/z ratio, migration time, and peak area.

Statistical analyses

Data are expressed as the means $\pm$ SD. Statistical analysis was performed using R software (version 3.4.2, <https://www.r-project.org/>). Principle component analysis (PCA) was carried out using multivariate techniques of metabolic global view by the R packages FactoMineR and factoextra. PCA components express a linear combination of the metabolite levels weighted by the component's leading values. Orthogonal partial least squares discriminant analysis (OPLS-DA) was also used to identify the metabolites factors that change before and after exercise using the R package ropls. A variable importance on projection (VIP) scores greater than 1.5 was used as the cut-off to select the most important metabolites responding to exercise for further analysis. To identify metabolites that were significantly changed by exercise, we used nonparametric Wilcoxon's signed-rank test. An estimate of the false discovery rate (FDR) was calculated to consider the multiple comparison that normally occurs in metabolomics-based studies, with $FDR < 0.5$ used as the cutoff for significance. For heatmap analysis, metabolites standardization was z-scaled by subtracting their means and followed by division by standard deviations. Relationship between saliva metabolites and fatigue VAS was analyzed by using a Pearson correlation coefficient, $p < 0.05$ for statistically significant.

Results

Table 1 shows subject characteristics and exhaustive exercise data. All subjects completed the exercise reaching an exhaustive fatigue state for an average duration of 1713 ± 191 s. Non-targeted metabolomics were applied to determine the 210 metabolites. PCA (Fig. 1) showed that principle components 1 and 2 captured together 40% of variance of the data, as well as the differential clustering before and after exercise. OPLS-DA modeling revealed a difference between pre- and post- exercise samples ($R^2Y = 0.929$, $Q^2Y = 0.596$) and identified 29 metabolites with VIP scores of 1.5 and higher (Table 2). These metabolites were attributed to carbohydrate metabolism, glycolysis, ketoacidosis, fatty acid metabolism, protein metabolism, and TCA cycle metabolism. These metabolites were differently clustered in the heatmap

before and after exercise (Fig. 2). Sixteen metabolites were significantly increased post-exercise compared to pre- exercise after adjusted FDR (Table 2). Furthermore, the fatigue VAS was significantly correlated only with an increase in cyclohexylamine (Fig. 3).

Table 1
The characteristics parameters during exercise exhaustion.

Variables			
Age (years)	29	±	3
Height (cm)	170	±	7
Weight (kg)	66	±	8
Parameters during exercise test			
Time to exhaustion (s)	1713	±	191
Maximum oxygen uptake (mL/kg/min)	52	±	6
Maximum ventilation (L/min)	144	±	23
Maximum heart rate (bpm)	186	±	13
Fatigue visual analog scale (mm)			
Pre	35	±	22
Post	83	±	12*
Data are shown as means ± SD. * $p < 0.05$ vs. Pre.			

Table 2

The identified salivary metabolites that changing abundance before and after exhaustive exercise

Metabolite	Related metabolic pathway		VIP	Ratio
Lactic acid	Glycolysis	Glycolysis metabolism	2.406	45.72*
Dihydroxyacetone phosphate	Carbohydrate metabolism	Glycerol phosphate shuttle	2.377	4.62*
Pyruvic acid	Carbohydrate metabolism	Carbohydrate metabolism	2.362	30.73*
2-Oxoisovaleric acid	Ketoacidosis	BCAA degradation	2.311	16.80*
2-Hydroxybutyric acid	Glycolysis	Propanoate metabolism	2.267	19.79*
Glycerol 3-phosphate	Carbohydrate metabolism	Glycerol phosphate shuttle	2.217	27.63*
Isethionic acid	Not available	Not available	2.100	6.08
4-Methyl-2-oxovaleric acid	Ketoacidosis	BCAA degradation	2.033	5.11*
2-Hydroxyvaleric acid	Glycolysis	Lactic acidosis	1.956	2.32*
Phosphorylcholine	Lipid, Fatty acid metabolism	Phospholipid biosynthesis	1.910	2.40
Phosphocreatine	Protein metabolism	Arginine and Proline metabolism	1.884	9.14*
Crotonic acid	Not available	Not available	1.813	3.34*
Dyphylline	Ketoacidosis	Xanthine derivative	1.718	4.64
Isocitric acid	TCA cycle metabolism	TCA cycle metabolism	1.714	3.89
Tyr-Arg_divalent	Not available	Not available	1.713	5.47
2-Oxoglutaric acid	TCA cycle metabolism	TCA cycle metabolism	1.698	8.52*
Creatinine	Protein metabolism	Creatine metabolism	1.692	1.43*
Alanine	Glycogenesis	Glucose-Alanine cycle	1.684	1.57*
Cyclohexylamine	Not available	Not available	1.666	NA ^a
Guanidoacetic acid	Protein metabolism	Arginine and Proline metabolism	1.628	1.61*
N6,N6,N6-Trimethyllysine	Lipid, Fatty Acid metabolism	Carnitine synthesis	1.612	7.00

VIP: Variable importance in the projection. ^a; The metabolite was not detected pre-exercise. * Significance change found between pre and post exercise after adjusted FDR.

Metabolite	Related metabolic pathway		VIP	Ratio
1-Methylhydantoin	Not available	Not available	1.580	1.31
cis-Aconitic acid	TCA cycle metabolism	TCA cycle metabolism	1.574	4.18
Saccharopine	Protein metabolism	Lysine degradation	1.550	2.48*
Butyric acid	Fatty acid metabolism	Fatty acid biosynthesis	1.546	1.92*
Malic acid	TCA cycle metabolism	TCA cycle metabolism	1.531	5.65
VIP: Variable importance in the projection. ^a ; The metabolite was not detected pre-exercise. * Significance change found between pre and post exercise after adjusted FDR.				

Discussion

In this study, we investigated the impact of exhaustive exercise on salivary metabolites and the relationship between fatigue and metabolites. The main findings of this study were as follows: first, exhaustive exercise led to changes in metabolic profile. Second, bioinformatic techniques including OPLS-DA analysis found that the 29 metabolites related to carbohydrate, fatty acid, protein, and TCA cycle metabolism were associated with the changes in metabolites after exercise. Furthermore, the changes in cyclohexylamine levels were significantly correlated with subjective fatigue. Therefore, these metabolites could play an important role in fatigue during exhaustive exercise.

The metabolomics approaches have revealed that acute exercise changed the metabolic profile associated with adenosine triphosphate production, including glycolysis (e.g., pyruvate and lactate), fatty acid oxidation (e.g., palmitate), protein metabolism (e.g., alanine), and ketone bodies (e.g., hydroxybutyrate).^[11,12] Consistent with previous studies, the present study found that exhaustive exercise increased these energy substrates related to carbohydrate, fatty acid, and amino acid metabolism (Table 2). These metabolic responses were depicted in principal components of the PCA scores plot (Fig. 2) accounting for about 40% of the total variance. Therefore, these results suggest that exhaustive exercise acutely changes the metabolic profile related to the energy production pathway.

Prolonged exhaustive exercise was associated with an increase in long chain fatty acids, medium chain fatty acids, fatty acid oxidation products, and ketone bodies.^[13] Nieman et al. (2017)^[14] have showed that these fatty acid metabolites changed after exhaustive running at 70% maximum oxygen uptake for a means duration of 2.26 h. Moreover, Manaf et al. (2018)^[7] have found changes in metabolites related to fatty acid, indole, neurotransmitter substances, and amino acid pathways after exhaustive cycling at an intensity of 3 mmol/L of lactate for a means of 1.2 h. The present study investigates the metabolomic profile response to a step-load cycling exercise until exhaustion (below 0.5 hours), a protocol that would be able to elicit central fatigue in a relatively short duration by high intensity exercise.^[8] The results of the present study demonstrated that our exhaustive exercise changes the metabolomic profile mainly

associated with glycolysis (e.g., lactate, pyruvate, dihydroxyacetone phosphate, and 2-hydroxyvaleric acid) and ketoacidosis (e.g., 2-oxoisovaleric acid and 4-methyl-2-oxovaleric acid). Therefore, metabolite response may be related to the nature of exercise factors such as duration and intensity.

Metabolite profile clustering was different before and after exhaustive exercise, and carbohydrate glucose metabolites showed increased VIP scores and fold changes compared to others (Table 2). In addition, TCA cycle intermediates, ketoacidosis, and some amino acid metabolites were associated with post-exercise metabolic profile changes, although most TCA cycle intermediates did not increase significantly. TCA cycle is the final pathway of carbohydrate, lipid, and some amino acid to produce energy via oxidative phosphorylation mainly during aerobic exercise. Taken together, the exhaustive exercise in the present study would reflect mostly anaerobic glycolysis metabolism, rather than the aerobic oxidation pathway, although both pathways were included in the exercise protocol. During relatively high intensity exercise, glucose metabolism is facilitated and pyruvate and lactate were increased. Under these conditions, pyruvate, lactate, and amino acid (alanine) are involved in restoration of glucose metabolism in the liver.^[15] Indeed, we observed that pyruvate, lactate, and alanine were increased post-exercise in this study, suggesting that the glucose metabolism pathway was accelerated at the end of the exercise.

In this study, the changes in fatigue VAS were correlated with an increase in cyclohexylamine within the VIP metabolites (Fig. 3). Cyclohexylamine is an acyclic aliphatic amine, and known as a metabolite produced by the artificial sweetener cyclamate.^[16] An early study has reported that ingestion of cyclohexylamine increased blood pressure and plasma free fatty acids, and that cyclohexylamine have a sympathomimetic capability.^[17] Animal studies demonstrated that cyclohexylamine was deaminated to the corresponding ketones by microsomes in the rabbit liver.^[18] Interestingly, metabolomics analysis in the present study could not detect cyclohexylamine at the baseline; however, cyclohexylamine increase was detected in several subjects after exercise. These findings may imply that exhaustive exercise-induced fatigue is in part mediated by cyclohexylamine. In this regard, further studies are necessary to elucidate a relationship between exercise and cyclohexylamine.

In conclusion, this study investigated the salivary metabolomic profile following exhaustive exercise-induced fatigue. We have identified energy-related metabolites that were significantly increased after exhaustive exercise. Our findings show an increase in metabolites related to glycolysis, lipolysis, amino acid metabolism, and ketone bodies. Furthermore, changes in cyclohexylamine levels were associated with an increase in fatigue. This metabolomic profile signature would serve as a pilot understanding of exercise induced fatigue.

Abbreviations

CE-TOFMS: capillary electrophoresis and time-of-flight mass spectrometry

FDR: false discovery rate

OBLA: onset of blood lactate accumulation

OPLS-DA: orthogonal partial least squares discriminant analysis

PCA: principle component analysis

TCA: tricarboxylic acid

VAS: visual analog scale

VIP: variable importance on projection

Declarations

Acknowledgement

We would thank all the participants for their valuable contribution to this study.

Competing interests

The authors declare no competing interests.

Author contribution

NA conception and design of the research; NA and MO data acquisition and analysis; NA and MN interpretation of the data; NA drafting the manuscript; MO and MN edited and revised manuscript. All authors read and approved the final version of manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participant

All methods were performed in accordance with Ethics Committee of the Japan Institute of Sports Sciences. All the participants were informed on the purpose of the study and written informed consent was obtained from each participant prior to participation.

Competing interests

The author declare that they have no competing interests.

Constant for publication

Not applicable.

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Figures

Figure 1

The principal component analyses (PCA) scores plot pre- and post-exercise.

Figure 2

The dendrogram heatmap of variable importance on projection metabolites pre- and post-exercise.

Figure 3

A relationship between the changes in fatigue visual analog scale and the increase in cyclohexylamine.