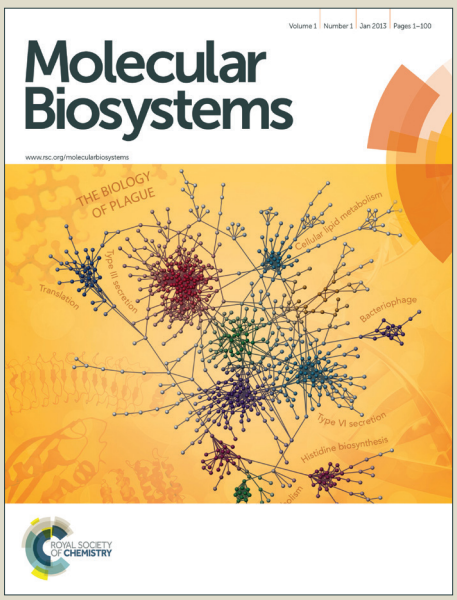
Molecular BioSystems

Accepted Manuscript



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Plasma metabolic signatures reveal regulatory effect of exercise training in *db/db* mice

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Abstract

Type 2 diabetes (T2DM) is caused by a complex set of interactions between genetic modifications and life styles. This complexity faces challenges for a full understanding of altered metabolic pathways contributing to the development of T2DM, awaiting a comprehensive metabolic analysis. Exercise training is a common therapeutic approach known to antagonize the metabolic consequences of T2DM. However, the metabolic phenotypes of exercise effect in T2DM have not been clearly characterized. Here, we present the effect of physical activity on biochemical changes in diabetic *db/db* mice. Untargeted metabolomics study based on liquid chromatography coupled with high resolution mass spectrometry was carried out to delineate plasma metabolic signatures in conjunction with multivariate statistical analysis. As a result, a total of 24 differential metabolites were identified, covering amino acids, organic acids and lipids. Three biomarkers, including lysine, creatine and uridine were significantly reversed by exercise training in *db/db* diabetic mice groups compared to lean *db/m+* groups. Of note, pantothenic acid and palmitoylcarnitine which involved in fatty acid β-oxidation (FAO) was promoted by exercise training in diabetic mice rather than lean mice. These findings indicated that diabetic mice might be more susceptible to exercise for energy expenditure. Together, the results might demonstrate that exercise could mitigate insulin resistance in T2DM through improving FAO and and uridine in blood might be an important indicator to reflect insulin sensitivity that promoted by exercise training on T2DM mice.

Key words: Type 2 diabetes mellitus (T2DM); fatty acid β-oxidation (FAO); uridine; metabolomics; LC-MS; insulin resistance; *db/db* mice

Introduction

Type 2 diabetes mellitus (T2DM) is one of the most common diseases with over 150 million patients worldwide [1, 2]. The disease causes an annual cost of 132 billion US dollars for the United States Healthcare System [3]. T2DM is closely associated with obesity [4-6], mainly characterized by insulin resistance and dysfunction of insulin secretion [7, 8]. Recently, the growing evidence on pathophysiologic mechanisms, such as increase in glucagon secretion, impaired ability of glucose reabsorption in renal, pancreas islet axis dysfunction, insulin resistance in brain and lipotoxicity, advances our understanding of this disease [9-11]. Therefore, T2DM is caused by a complex set of pathogenesis. This complexity faces changes for a full understanding of molecular metabolic pathways that contribute to the development of T2DM and adds difficulty for the prevention and treatment of the disease.

For decades, exercise along with diet and medication, has been considered as one of the effective approach of diabetes management [12-14]. Regular exercise training is highly recommended for patients with T2DM since physical activity may have beneficial effects on regulating metabolic dysfunction during the development of diabetes complications [15]. Lots of studies have been carried out to investigate the mechanism of exercise on T2DM [16-19]. However, the impingement of physical activity on metabolism in T2DM has not been fully understood so far.

Process in investigating the regulatory effect of physical activity on multi-pathogenesis disease (T2DM) can be aided by recent advanced technologies for comprehensive metabolic analysis, which generally termed as “metabolomics”. Metabolomics is a quantitative assessment of small, low molecular weight, endogenous metabolites in response to physiological or pathophysiological stimuli [20, 21]. It allows simultaneous quantification of numerous metabolites in the same small sample volume prepared from any type of samples (such as body fluid and various types of tissue) [22]. In recent studies, metabolomics has been used to investigate the differences between insulin resistant and sensitive patients with obesity [23], or to compare metabolic changes between lean and diabetic individuals [24]. Jane Sharer and colleagues [18] used metabolomics approach to investigate metabolic response to exercise treatment in lean and diet-induced obese mice. Therefore, this unbiased, systematic method is powerful in finding out whether physical activity alters the basal metabolite profile and corrects T2DM induced shifts in metabolic spectra.

In this study, we developed and applied liquid chromatography mass spectrometry (LC-MS)-based metabolomics method to study the regulatory effect of exercise training on *db/db* diabetic mice. The *db/db* mouse (also relabeled as *lepr^{db}*) is derived from an autosomal recessive mutation that is traced to *db* gene on chromosome 4 on C57BL/KsJ mouse. The *db* gene was encoded for leptin receptors [25, 26]. Lack of functioning leptin receptor will result in failure to elicit effect on food intake, leading to obesity, insulin resistance, dyslipidemia and hyperglycemia. The *db/db* mice have been extensively used for the study of T2DM metabolic dysfunction and insulin activity to a variety of agents [27, 28]. In the current study, a number of biomarkers have been identified. When comparing regulatory effects on *db/db* diabetic mice and *db/m+* normal mice, it was found that physical activity might have positive regulatory effect on the dysfunction of energy metabolism that is induced by T2DM, particularly fatty acid catabolism, as well as improvement of insulin sensitivity.

Experiment

Material and chemicals

HPLC grade methanol was purchased from RIC Labscan Ltd. Co., (Bangkok, Thailand). HPLC grade Acetonitrile was purchased from Tedia Company (Fairfield, OH, USA). Formic acid (FA) was purchased from Sigma - Aldrich (St. Louis, MO, USA). ACQUITY UPLC HSS T3 column (100 × 2.1 mm i.d., 1.7 µm particle size) (Waters, Milford, MA) was selected in this study. Authentic standards of L-lysine, L-proline, L-arginine, acetylcarnitine, palmitoylcarnitine, L-carnitine, glutamine, L-tyrosine, fumaric acid, pantothenic acid, creatine and uridine were purchased from Sigma- Aldrich (St. Louis, MO, USA). Pure water was prepared from a Milli-Q Ultrapure water system (Millipore, Billerica, MA, USA). All other reagents and chemicals used in this experiment were of analytical grade.

Animal experiments

For this study, the C57BL/KsJ *db/db* male mouse was used as type 2 diabetes mellitus (T2DM) model mouse, and C57BL/KsJ male *db/m+* mouse was used as control mouse. Each type of mouse was also randomly assigned to either sedentary and exercise treated groups. The *db/db* mouse group treated with sedentary (*db/db* sedentary (OS) group, n = 8); *db/db* mouse group treated with exercise (*db/db* exercise (OE) group, n = 8); *db/m+* mouse group treated with sedentary (*db/m+* sedentary (LS) group, n = 9); *db/m+* mouse group treated with exercise (*db/m+* sedentary (LE) group, n = 9). All animal experimental procedures were conformed and approved by local ethics committee, the Universite´ catholique de Louvain

and the Rangueil Hospital animal ethics committee. C57BL/KsJ *db/db* mice and their controls (*db/m+* mice) were maintained in a specific pathogen-free (SPF) colony (inverted 12 hours daylight cycle and lights-off at 10:00 a.m.), fed standard laboratory chow (mouse No. 3 Breeding, Special Diets Services) and *ad libitum* access to water and food [29]. Two exercise groups (LE and OE groups) were treated under moderate intensity: run at 8 m/min for 30 min in the morning on the treadmill (Columbus Instruments) per day, 6-day running period each week consecutively for 4 weeks. In order to account for stress induced by physical activity, sedentary animals were also put in a stationary treadmill for acclimation. At the end of exercise period, animals rested for 24 h to minimize any direct effects of physical activity [30]. Then all the animals were weighed. After anesthetized with pentobarbital, whole blood was collected to a 1.5 mL micro-centrifuge tube moistened by heparin sodium. The blood samples were centrifuged at 13,200g for 10 min (4 °C), transfer the supernatant to a new 1.5 mL micro-centrifuge tube, and stored at −80 °C before analysis. All blood samples were collected between 8:00 a.m. to 10:00 a.m. after an overnight fast. Blood glucose was assessed with a glucose meter (Roche Diagnostics, Meylan, France) on 3.5 µl blood. In addition, the concentration of insulin was also assessed described as previously [31].

Sample preparation

The plasma samples were thawed before analysis and 50 µL aliquots of plasma were added to 200 µL methanol. The mixture was vortexed for 1 min, and then centrifuged at 13,200 rpm for 10 min at 4 °C. The supernatant was removed and dried under gentle nitrogen stream, and stored at –80 °C before re-dissolving in 50% methanol (methanol/water (v/v) = 1:1) for analysis and at a random order. Meanwhile, a quality control (QC) sample was made by pooling the same volume (10 µL) of each re-dissolved plasma sample. The QC sample was also operated randomly throughout the experiment to monitor instrument stability in terms of masses, retention time and intensities. A blank sample (acetonitrile) was injected every 3 sample injections to minimize the carry-over throughout the sequence analysis. Stock solution was first prepared in methanol, and then diluted to a proper concentration for future verification analysis.

Instrumental analysis

The analyses were performed on a Thermo Scientific quaternary Accela 1250 pump with a PAL Sample Manager coupled to a LTQ Orbitrap TM XL mass spectrometer (Thermo Fisher Scientific, MA, USA). The column oven was maintained at 40 °C, and the flow rate was set at 0.4 mL min^{-1}. The mobile phases were ultrapure water (A) with 0.1% formic acid and acetonitrile (B) with 0.1% formic acid. The experiment was operated in both positive and negative ion modes. The sample injection volume was 10 µL in positive ion mode and 8 µL in negative ion mode. The elution gradient was shown in supplementary table 1. The parameters of mass detection were set as follows: Heater Temp.: 350 °C; Sheath Gas Flow Rate: 50 arb; Aux Gas Flow Rate: 5 arb; I Spray Voltage: 3 kV; Capillary Temp.: 300 °C; Capillary Voltage: 50 V and Tube Lens: 80.00; the mass scan range was *m/z* 100 - 1000. The MS/MS analysis was acquired in targeted MS/MS mode with 3 collision energies of 10 eV, 20 eV and 40eV, respectively.

Quality control

The stability and repeatability of this study were measured for the assessment of LC-MS approach. According to different polarities and masses (*m/z*), 5 ions have been extracted for validation according to the variation of their peak intensities and retention times. The data of QC samples were used for the evaluation of stability and repeatability of the large-scale sequence analysis. Stability was assessed by 6 QC samples injected throughout the sequence analysis, while repeatability was assessed by injecting QC samples continuously for 6 times. The retention time and peak intensity of the 5 extracted ions were then involved to analyze the variation.

Data processing and multivariate statistical analysis

The raw LC - MS data files were initially converted into mzXML format files. The program of XCMS [32] (https://xcmsonline.scripps.edu/) has been implemented in R language for retention time alignment and peak intensity extraction in each chromatogram. A table was generated including the information of *m/z*, retention time and ion intensity. The ion intensities were imported into SIMCA-P software (Ver. 11, Umetrics, Umea, Sweden) for multivariate analysis. Partial least squares Discriminant Analysis (PLS-DA) and Principle Component Analysis (PCA) were used for multivariate data analysis. Comparison of the metabolome was carried out between LS and OS groups. It should be noted that quality control for metabolic profiling analysis was performed prior to sample analysis. One-way analyses of variance (ANOVAs) with a Bonferroni correction of the SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) were used for significance analysis. Differences were considered statistically significance with the threshold of $P < 0.05$.

Results

Physiological and exercise performance of mice

In *db/db* mice, leptin resistance results in rapid gain in body weight. As was shown in Fig. 1a, the average weight in *db/db* sedentary (OS) group and *db/db* exercise training (OE) group were significantly increased when compared with *db/m+* sedentary (LS) group. However, there was not any significant regulatory effect by exercise training on both *db/db* and *db/m+* mice groups in weight loss. Besides, we all know that the increased blood glucose level is an important indicator of diabetes, while insulin is the principal hormone in regulating the uptake of glucose from blood into cells of the body [33]. In this study, blood glucose levels were examined on *db/m+* and *db/db* mice regardless of treatment of sedentary or physical activity intervention. We can see from Fig. 1b, the level of blood glucose was significantly increased in obesity mice groups (OS and OE) when compared with lean mice groups (LS and LE). However, physical activity did not show regulatory effect on increased blood glucose level on obese mice. Oral glucose tolerance test (OGTT) was also carried out, we can see from Fig. 1c that after oral glucose injection, the blood glucose level (120 min) has been significantly increased in OS and OE groups (Fig. 1c)), but did not account for statistical significance between the two groups after exercise training. However, the results of insulin tolerance test (ITT) showed a significant decrease in blood glucose level (120 min) on *db/db* mice after exercise training when compared with OS group (Fig. 1d), indicating that physical activity might have the ability to help to improve insulin resistance. All results of physiological characteristics indicated that the model of diabetes (*db/db*) was successful and exercise training might get positive regulatory effects on insulin resistance on diabetic mice.

Method validation of LC-MS analysis

To assess system reliability throughout sequencing analysis on LC-MS, stability and repeatability was performed (Supplementary table 2). For the validation of system stability, the relative standard derivations (RSDs) of these 5 ions were less than 1.28% for retention times and 7.66% for peak intensities. For the validation of system repeatability, the RSDs of selected 5 ions were less than 1.36% for retention time and 7.78% for peak area intensities. The data demonstrated that the stability and repeatability of this proposed method was satisfactory for the large-scale sample sequence analysis.

Multivariate statistical profiling analysis

In this study, multivariate statistical analysis method Partial Least Squares Discriminant Analysis (PLS-DA) was applied in metabolic profiling analysis for biomarker identification related to the development of T2DM. R^2X, R^2Y and Q^2 (cum) are important parameters to evaluate the PLS-DA model. In order to acquire a high predictive ability of the model and prevent the model from over-fitting, the value of the three parameters should be close to 1. The established PLS-DA model in this study using cross validation could describe 84.0% of the variation in X (R^2X = 0.84), 99.0% of variation in Y (R^2Y = 0.99), and with a predictive ability of 86.6% (Q^2 (cum) = 86.6%) in negative ion mode. Meanwhile, the value of R^2X, R^2Y and Q^2 (cum) were 0.72, 1.00 and 0.992 respectively, in positive ion mode. All the parameters indicated that the established models obtained satisfactory grouping and predicating ability.

Identification of biomarkers related to T2DM and exercise training

Based on PLS-DA analysis (See Fig. 2), detected ions (1315 ions in negative ionization mode and 1392 ions in positive ionization mode) which contributed to the discrimination and clustering were selected according to the threshold of variable importance in the projection (VIP) values (VIP > 1.5). At last, 24 features (15 ions in positive ion mode and 9 ions in negative ion mode) were identified by searching MS and MS/MS fragments in Biofluid Metabolites Database (http://metlin.scripps.edu) and Human Metabolome Database (HMDB) (http://www.hmdb.ca), and part of them were confirmed by authentic standards (Including L-lysine, glutamine, L-proline, L-valine, L-arginine, L-tyrosine, malic acid, L-palmitoylcarnitine, pantothenic acid, fumaric acid and uridine). The detailed information of identified biomarkers was listed in Table 1 and Table 2. These biomarkers were mainly classified into amino acids and their derivatives, organic acids and lipids. Three biomarkers, including creatine, uridine and lysine (Fig. 3) were successfully reversed by exercise training that induced by T2DM. Interestingly, the concentration of pantothenic acid, palmitoylcarnitine and hydroxyphenyllactic acid (Fig. 4) were specifically and significantly increased after physical activity on diabetic mice.

Discussion

Multivariate analysis methods PLS-DA and Principle Component Analysis (PCA) have been applied to give us an overview of the regulatory effect of exercise training on different types of mice. As we can see in Fig. 2 and supplementary Fig. 2, *db/db* diabetic mice groups were clustered far away from *db/m+* mice groups in both positive and negative ion modes regardless of the treatment of sedentary and exercise training, indicating that there were notable metabolic changes between the two types of mice (See Fig. 2 and supplementary Fig. 2(N1, N2, P1 and P2)). When assessing the regulatory effect of exercise training on the two types of mice, we found that LE group was overlapped with LS group in both positive and negative ion modes (See Fig. 2 and supplementary Fig. N3 and Fig. P3), showing that the metabolic change was small between the two groups, and indicating that exercise rarely had regulatory effects on lean mice (*db/m+* mice). However,

OE group can be clearly separated from OS group in both ionization modes (See Fig. 2 and supplementary Fig. N4 and P4), suggesting that post-exercise impingement might affect body metabolism in diabetic mice. Furthermore, when evaluating the metabolomic alterations of diabetes based on identified biomarkers (See Table 2), we found that 20 identified biomarkers were significantly changed between LS and OS groups. Meanwhile, when evaluating the metabolomic alterations of exercise training on *db/m+* and *db/db* mice, we found that only one of the Branched-chain amino acids (BCAAs), valine, was significantly changed after exercise training on *db/m+* mice, which in accordance with the findings that the concentration of valine were lower after short-term acute exercise training in human skeletal muscle [34]. However, there isn't significant changes of valine between OS and LS group, indicating that the changes of valine might not related to diabetes, but related to exercise regardless of control mice or diabetic mice. Interestingly, 9 biomarkers, including hippuric acid, DL-2-aminooctanoic acid, hydroxyphenyllactic acid, lysoPC(16:0), L-palmitoylcarnitine, uridine, pantothenic acid, L-lysine, and creatine were significantly changed after exercise training on diabetic mice.

Here we detected creatine (See Fig. 3B). Indeed, creatine generally behaves as phosphocreatine (PCr) and has the ability to increase the stores of PCr, potentially through increasing the ability of muscle to regenerate ATP from ADP to match energy demands. In this study, we found that the concentration of creatine was decreased ($p < 0.05$) in OS group when compared with LS group. The observation was in accordance with the findings by Gavin E. Duggan et al. [18], who found that the level of creatine was also significantly decreased in obesity mice under high fat diet. The results indicated that decreased concentration of creatine might cause shorter storage of PCr in muscle and might further cause accumulation of fatty acids which contribute to body fat mass [35, 36]. Interestingly, it was found that exercise could significantly reverse the decreased concentration of creatine to normal level (LS group), indicating that moderate exercise might have good reverse effects on the dysfunction of energy metabolism that induced by diabetes.

Uridine, which contributes to the intracellular uridine diphosphate/uridine triphosphate (UDP/UTP) pool, is involved in important enzymes during the process of glycogen synthesis. In glycogenesis (See Fig. 3), glucose is firstly phosphorylated to glucose-6-phosphate (Gluc-6P) under participation of adenosine triphosphate (ATP) and converted to glucose-l-phosphate (Glucose-1P). Then, uridine diphosphoglucose (UDP-glucose) will be formed from glucose-1-P and UDP. In the final step, glycogen is generated from UDP-glucose together with the release of UDP. Since oral administration of glucose will increase the plasma concentration of uridine [37], it is suggested that UDP which is released from UDP-glucose, increases sharply along with abrupt decrease of UDP-glucose [38, 39] through glycogenesis process. Luciano Rossetti and colleagues [40] found that UDP-glucose depletion was effective in inducing skeletal muscle insulin resistance by infusion of uridine alone. In other words, extracellular uridine enhances UDP-glucose accumulation and reduces insulin sensitivity. In our study, it was found that the concentration of uridine in plasma was increased in OS group, indicating that *db/db* mice might induce insulin resistance through dysfunction of uridine metabolism. It is likely that exercise training could significantly increase plasma uridine to normal level by comparing OE group with LS group, indicating that uridine in blood might be an important indicator to reflect insulin sensitivity promoted by exercise training in diabetic mice.

Additionally, the induction of insulin resistance is induced by dysfunction of glucose, lipid metabolism and its metabolic sequelae. Of particular importance is that fatty acids can be metabolized via mitochondrial fatty acid β-oxidation (FAO) to prevent insulin resistance [41]. During this process, coenzyme A (CoA) plays an important role in tricarboxylic acid (TCA) cycle at the beginning of this pathway. Pantothenic acid, which is required in the formation of CoA, plays a critical role in the metabolism and synthesis of proteins, carbohydrates, and fatty acids [42, 43]. However, FA needs to be activated before binding to carnitine to generate “acylcarnitine” to enter the inner membrane of mitochondrial (IMM) for β-oxidation. Firstly, the free FAs in cytosol are bonded to CoA to form acyl CoA. Secondly, acyl CoA is transferred to carnitine molecule and combined with hydroxyl group of carnitine by carnitine acyltransferase I (CPTI) to form acylcarnitine on the outer side of mitochondrial. Thirdly, acylcarnitine will transport through carnitine-acylcarnitine translocase inside the mitrochondrial matrix. Finally, with the help of carnitine acyltransferase II (CPTII) located on the inner side of mitrochondrial membrane, acylcarnitine is converted to acyl CoA and free FAs which will be metabolized through β-oxidation to obtain usable energy (Adenosine triphosphate (ATP)) through the TCA cycle. Pantothenate Kinase (PanK) is the rate controlling step at the beginning of the biosynthesis of CoA through the formation of 4’-phosphopantothenate (4P-Pan) [44] from pantothenic acid. In this study, we have detected that the concentration of pantothenic acid (See Fig. 4A and Fig. 5) required to form CoA has been dramatically increased after exercise training between T2DM groups (OE vs OS) but not between lean control mice groups (LE vs LS), indicating that diabetic mice might be more sensitive to physical activity than lean control mice for FA β-oxidation through increasing the concentration of obligatory activator of CoA. Interestingly, the regulatory effect of pantothenic acid on obese patients in plasma were also significantly increased after exercise training [45], which in accordance with our hypothesis that physical activity might have good regulatory effect on improving the efficiency of FAO. Furthermore, worth mentioning is palmitoylcarnitine (C16), Roberta Leonarni and colleagues [46] have reported that

palmitoylcarnitine plays a positive role in the reversal of acetyl-CoA inhibition of PanK2. In this study, the concentration of palmitoylcarnitine was significantly increased only in diabetic mice group by exercise training but not in lean mice group. Combined with the phenomenon of regulatory effect of pantothenic acid, it is indicated that diabetic mice might be more susceptible than lean control mice in improving FA β-oxidation to meet the increased energy demands during exercise training. However, many studies have also reported that the increased concentration of palmitoylcarnitine could also play a role in cytotoxicity in Caco 2 and IEC-18 cells [47], indicating that in addition to good regulatory effect on diabetic mice through improving the efficiency of FAO, exercise training might also cause cytotoxicity specifically on diabetic mice.

Besides, we all know that lysine and methionine provide the carbon backbone for carnitine. Randall S. *et al*. have found that an dietary supplementation of lysine could lower the concentration of free carnitine in rats' plasma [48]. Klaus Eder and colleagues [49] also reported that high lysine diet had lower concentration of total and free carnitine in plasma, kidney, liver and skeletal muscle in pigs. In this study, the concentration of lysine was significantly lower in OS group when compared with *db/m+* mice groups (Fig. 3), indicating that the decreased concentration of lysine might associate with a reduction of free carnitine which plays a critical role of transportation of FA into mitochondrial matrix to yield energy through β-oxidation. However, the concentration of lysine was significantly increased when compared with OS group after exercise training on diabetic mice, indicating that physical activity might also get positive effect on regulating FA β-oxidation via carnitine metabolism pathway.

Furthermore, Hydroxyphenyllactic acid [Fig. 4C], metabolite of tyrosine, has been shown that may function as a natural anti-oxidant by decreasing reactive oxygen species (ROS) production in mitochondria [50]. In this study, the concentration of hydroxyphenyllactic acid has been dramatically increased (5.07 fold change, OE *vs* OS (See Supplementary Table 1)) specifically in diabetic mice group after exercise training, indicating that diabetic mice might be more susceptible than *db/m+* mice in decreasing mitochondria ROS production during exercise training.

In summary, our results presented that moderate exercise training might got positive regulatory effect in improving energy metabolism and insulin sensitivity. However, the results also showed an altered fatty acid β-oxidation in mitochondrial defect [51, 52] and the dysfunction of mitochondrial ROS production. This could be that a greater length of time, along with a more intense exercise of training that might be needed in our future experiment to particularly investigate whether the defect of β-oxidation in T2DM mice can be improved.

Conclusions

Untargeted metabolomics profiling of plasma from *db/db* diabetic mice with and without exercise revealed that physical activity might have positive regulatory effects on energy metabolism associated with creatine/phosphocreatine system. Of note, the level of plasma uridine was also reversed by exercise training in a good agreement with the change of plasma insulin level, which indicated that uridine in blood might be an important indicator to reflect insulin sensitivity that promoted by exercise training. Meanwhile, FA catabolism through β-oxidation was more susceptible to physical activity on diabetic mice than lean mice. These metabolic markers demonstrated in plasma could support our notion that physical activity did have positive impacts on T2DM mice and advance our understanding of the underlying mechanism linked to insulin sensitivity.

Acknowledgement

The authors would like to acknowledge the financial support from RGC-CRF (CUHK2/CRF/12G).

Figure legends

Table 1 Detailed information of identified biomarkers in mice plasma.

Table 2 Detailed information of regulatory effects of exercise on different types of mice.

Fig. 1 Physiological characteristics results of *db/db* and *db/m+* mice. Including body weight (a), blood glucose level (b), blood glucose level after glucose injection (OGTT) (c) and blood glucose level after insulin injection (ITT) (d).

Fig. 2 PLS-DA score plots of regulatory effects of exercise training on *db/m+* and *db/db* mice. (a) PLS-DA score plot in negative ionization mode; (b) PLS-DA score plot in positive ionization mode.

Fig. 3 Three biomarkers (including creatine, L-lysine and uridine) that have been significantly reversed by exercise training.

Fig. 4 Regulatory effect of exercise training on pantothenic acid, palmitoylcarnitine and hydroxyphenyllactic acid.

Fig. 5 Regulation pathways and regulatory effect of exercise training based on identified biomarkers.

Table 1 Biomarkers identified in mice plasma.

No.	Name	m/z	R.T.	Errors (ppm)	Ion modes	Fragment ions	Metabolites category
1	L-Lysine [a]	147.1125	0.60	0	positive	130.0826; 84.0808	Amino acids and derivatives
2	Glutamine[a]	145.0621	0.68	1	negative	127.0514; 109.0410	
3	L-Proline [a]	116.0711	0.75	4	positive	70.0650	
4	L-Valine [a]	118.0863	0.79	0	positive	72.0807;55.0541	
5	Creatine [b]	132.0766	0.96	0	positive	114.0658; 90.0549	
6	L-Arginine [a]	175.1187	1.11	0	positive	158.0922; 157.1082; 70.0650	
7	4-Guanidinobutanoic acid [b]	146.0924	1.42	0	positive	128.0817; 87.0440	
8	L-Tyrosine [a]	182.0812	2.92	0	positive	165.0547; 136.0753; 123.0440	
9	Hippuric acid [b]	178.0508	6.33	0	negative	134.0611	
10	N-Acetylleucine [b]	172.0978	6.78	0	negative	130.0872	
11	DL-2-Aminooctanoic acid [b]	160.1333	7.40	0	positive	114.1278	
12	Acetyl-tryptophan [b]	247.1080	9.19	1	positive	229.0973; 205.0965; 201.1023; 188.0708	
13	Malic acid [a]	133.0141	0.79	1	negative	115.0036; 71.0137	Organic acids
14	Hydroxyphenyllactic acid [b]	181.0505	5.76	0	negative	163.0400; 135.0449	
15	Suberic acid [b]	173.0818	7.04	0	negative	111.0815	
16	12-HETE [b]	319.2274	13.95	1	negative	301.2170; 179.1071	Lipids
17	L-Palmitoylcarnitine [a]	400.3430	17.78	0	positive	341.2688; 239.2371	
18	LysoPC(16:0) [b]	496.3410	17.25	0	positive	478.3309; 184.0738	
19	LysoPC(20:0) [b]	552.4030	20.81	0	positive	534.3923; 184.0735	
20	choline [b]	104.1068	0.72	1	positive	60.0819	
21	Fumaric acid [a]	115.0036	0.80	0	negative	71.0138	
22	Uridine [a]	243.0617	2.76	0	negative	200.0560; 110.0243	
23	p-Coumaric acid [b]	165.0546	2.92	0	positive	147.0440; 119.0491; 91.0543	
24	Pantothenic acid [a]	220.1182	5.59	1	positive	202.1075; 184.0969; 90.0549	

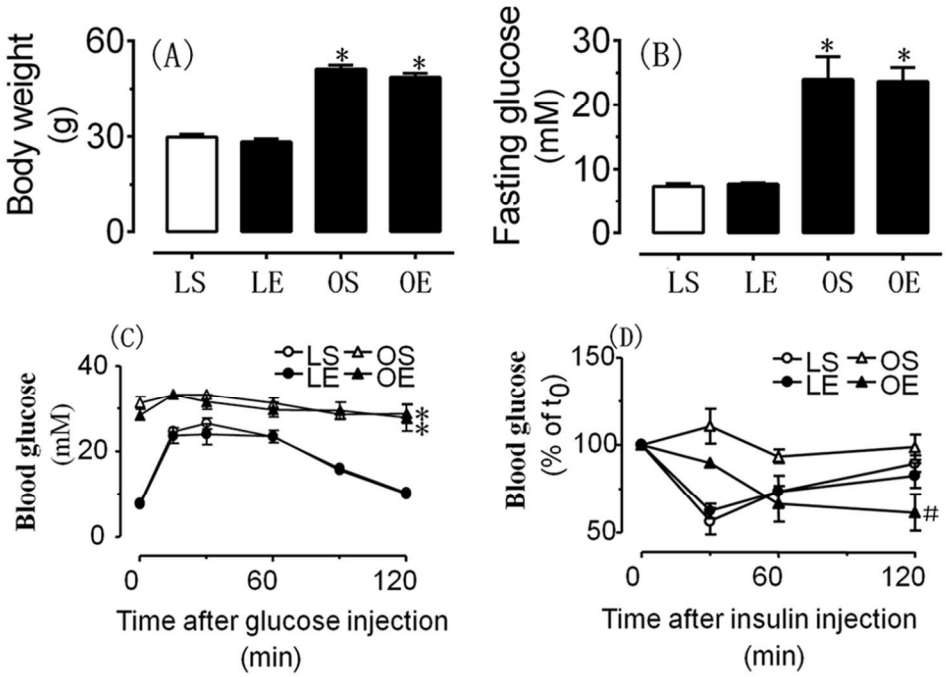
Note: [a] represents metabolites that identified by authentic standards; [b] represents metabolites that identified by accurate MSMS spectrum.

Table 2 Detailed information of regulatory effects of exercise on different types of mice.

Metabolites	LE vs LS			OS vs LS			OE vs OS			OE vs LE		
	Regulation trend	FC	P value	Regulation trend	FC	P value	Regulation trend	FC	P value	Regulation trend	FC	P value
L-Lysine	↑	1.07	0.58	↓	0.50	$1.79*10^{-4}$	↑	1.47	0.05	↓	0.68	0.02
Glutamine	↑	1.03	0.73	↓	0.58	$3.50*10^{-4}$	↑	1.54	0.61	↓	0.63	$2.97*10^{-4}$
L-Proline	↓	0.91	0.55	↓	0.72	0.03	↑	1.00	0.89	↓	0.76	0.19
L-Valine	↓	0.014	0.035	↓	0.069	0.178	↑	1.07	0.431	↑	5.37	0.016
Creatine	↓	0.94	0.51	↓	0.67	0.004	↑	1.94	0.03	↑	1.39	0.15
L-Arginine	↑	1.01	0.93	↓	0.83	0.25	↓	0.65	0.08	↓	0.53	0.03
4-Guanidinobutanoic acid	↓	0.89	0.72	↑	3.06	0.02	↑	1.02	0.95	↑	3.49	0.005
L-Tyrosine	↓	0.91	0.70	↓	0.59	0.05	↑	1.00	0.79	↓	0.70	0.23
Hippuric acid	↑	1.15	0.17	↑	2.75	$1.39*10^{-6}$	↓	0.60	0.046	↑	1.44	0.12
N-Acetylleucine	↑	1.04	0.83	↑	4.81	$1.43*10^{-4}$	↓	1.00	0.70	↑	5.20	0.005
DL-2-Aminooctanoic acid	↓	0.75	0.02	↓	0.74	0.004	↓	0.58	0.01	↓	0.57	0.002
N-Acetyl-DL-tryptophan	↑	1.11	0.48	↑	2.41	$8.26*10^{-4}$	↓	0.50	0.23	↑	1.43	0.38
Malic acid	↓	0.88	0.48	↑	2.06	0.001	↑	1.08	0.78	↑	2.52	0.003
Hydroxyphenyllactic acid	↓	0.81	0.10	↑	1.63	0.003	↑	5.07	0.05	↑	6.23	0.002
Suberic acid	↓	0.93	0.63	↑	1.54	0.01	↑	2.79	0.17	↑	2.97	0.01
12-HETE	↓	0.98	0.95	↑	3.03	0.007	↑	1.48	0.28	↑	4.54	$8.43*10^{-5}$
L-Palmitoylcarnitine	↓	0.80	0.32	↑	1.11	0.66	↑	7.09	0.02	↑	9.85	0.004
LysoPC(16:0)	↓	0.78	0.20	↑	1.25	0.93	↑	1.97	0.006	↑	3.15	$2.52*10^{-5}$
LysoPC(20:0)	↓	0.99	0.95	↓	0.16	$4.14*10^{-6}$	↓	0.65	0.63	↓	0.10	$3.84*10^{-7}$
Choline	↓	0.99	0.89	↓	0.39	$1.25*10^{-8}$	↑	1.43	0.20	↓	0.57	0.01
Fumaric acid	↑	1.02	0.91	↑	3.28	$3.08*10^{-5}$	↑	4.09	0.36	↑	4.01	$2.89*10^{-5}$
Uridine	↑	1.10	0.77	↑	1.98	0.003	↓	0.51	0.05	↓	0.93	0.868
p-Coumaric acid	↓	0.78	0.40	↓	0.47	0.008	↑	1.28	0.41	↓	0.78	0.46
Pantothenic Acid	↑	0.91	0.36	↑	2.32	$8.07*10^{-5}$	↑	1.65	0.03	↑	3.45	$1.06*10^{-4}$

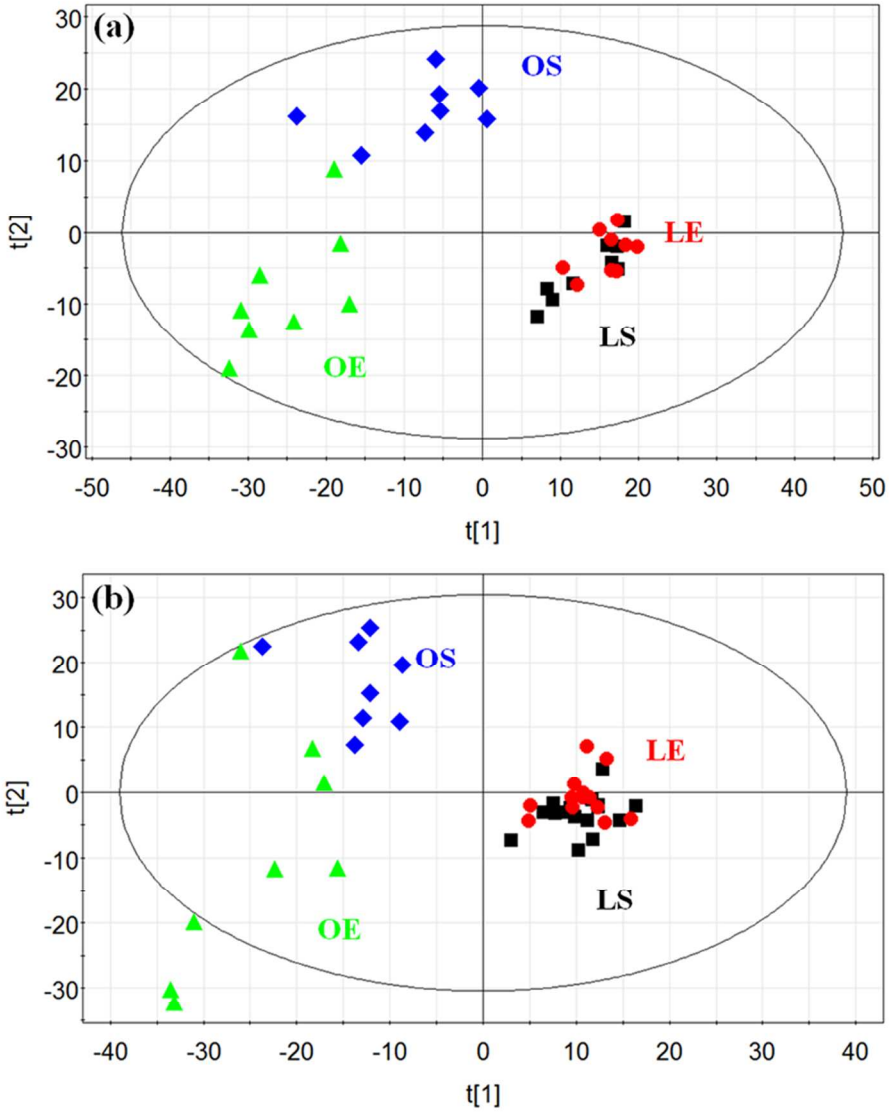
Note: FC represents fold change. ↑: Represents increased metabolites; ↓: Represents decreased metabolites.

Fig. 1 Physiological characteristics of *db/db* and *db/m+* mice.



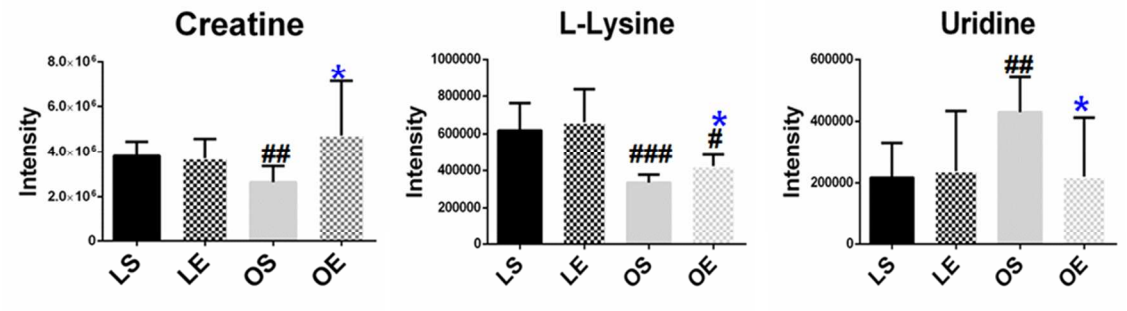
Note: LS: lean sedentary (*db/m*$^{+}$); LE: lean exercise (*db/m*$^{+}$); OS: obese sedentary (*db/db*); OE: obese exercise (*db/db*); * represents $p < 0.05$ vs LS; # represents $p < 0.05$ vs OS. (A) Body weight of four groups; (B) Fasting blood glucose level of four groups; (C) Oral glucose tolerance test (OGTT); (D) Insulin tolerance test (ITT).

Fig. 2 PLS-DA score plots of regulatory effects of exercise training on *db/m+* and *db/db* mice.



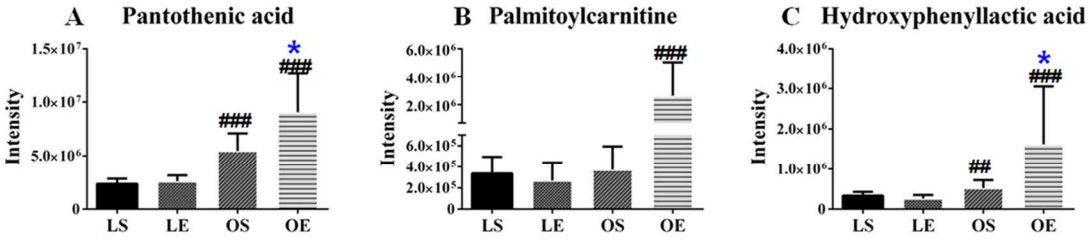
Note: (a) PLS-DA score plot in negative ionization mode; (b) PLS-DA score plot in positive ionization mode. LS: *db/m+* mice treated with sedentary, black square; LE: *db/m+* mice treated with exercise training, red dot; OS: *db/db* mice treated with sedentary, blue diamond; OE: *db/db* mice treated with exercise training, green triangle.

Fig. 3 Biomarkers that been significantly reversed by exercise.



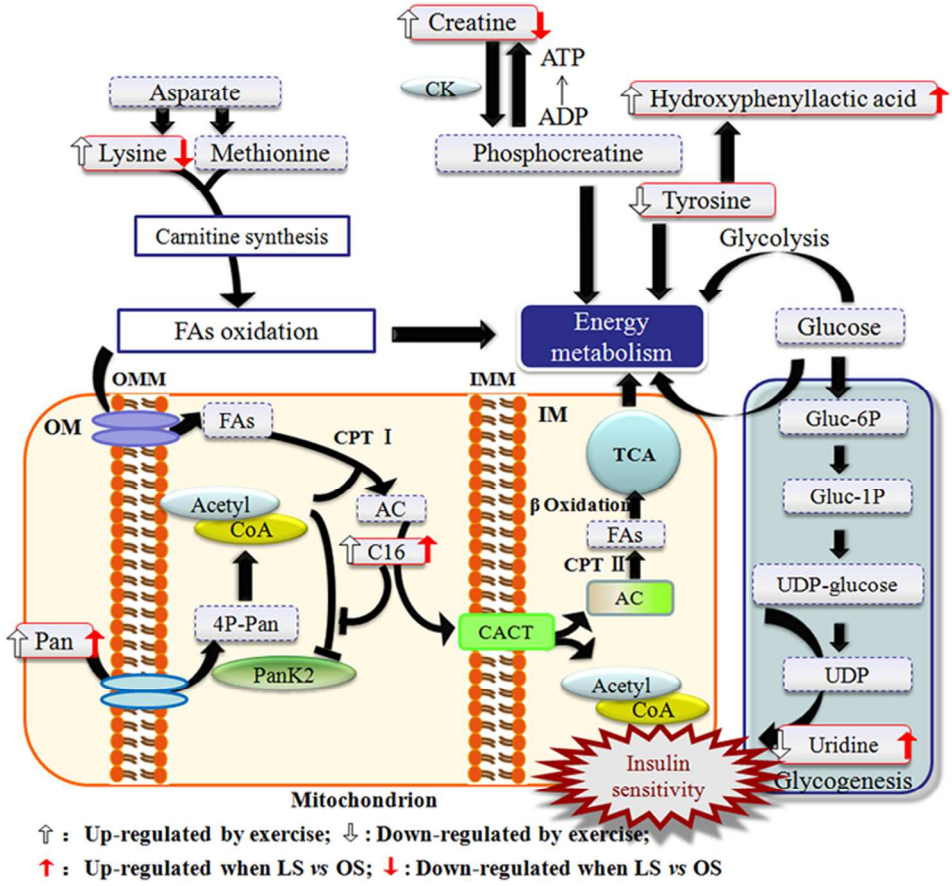
Note: The sample amount of each group used for analysis is 9, 9, 8 and 8 in LS, LE, OS and OE groups respectively. #: P value < 0.05, OS *vs* LS; ##: P value < 0.01, OS *vs* LS; ###: P value < 0.001, OS *vs* LS; * P value < 0.05, OE *vs* OS.

Fig. 4 Regulatory effects of exercise on pantothenic acid, palmitoylcarnitine and hydroxyphenyllactic acid.



Note: The sample amount of each group used for analysis is 9, 9, 8 and 8 in LS, LE, OS and OE groups respectively. #: P value < 0.05, OS or OE *vs* LS; ##: P value < 0.01, OS or OE *vs* LS; ###: P value < 0.001, OS or OE *vs* LS; * P value < 0.05, OE *vs* OS.

Fig. 5 Proposed metabolic pathways regulated by exercise training based on identified biomarkers.



Note: Pan: pantothenic acid; 4P-Pan: 4'-phosphopantothenate; Pank2: pantothenate kinase 2; FAs: fatty acids; AC: acylcarnitines; C16: palmitoylcarnitine; CPT1: Carnitine palmitoyltransferase I, CPT II: Carnitine palmitoyltransferase II; CACT: carnitine-acylcarnitine translocase; TCA: Citric acid cycle; CK: Creatine Kinase; ATP: Adenosine triphosphate; ADP: adenosine diphosphate; UDP: Uridine diphosphate.Gluc-6P: glucose-6-phosphate; Gluc-1P: glucose-l-phosphate; OM: outside of the mitochondrial; IM: inner side of the mitochondrial; OMM: outside membrane of mitochondrial; IMM: inner side membrane of mitochondrial.

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