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Effect of metformin on the urinary metabolites of diet-induced-obese mice studied by ultra performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOF/MS)

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ABSTRACT

Obesity is becoming a health concern worldwide and metformin, a first line anti-diabetic drug, was associated with weight loss under different backgrounds. However, most researches focused on the anti-diabetic mechanism and less attention has been paid on the mechanism of weight loss of metformin. Therefore, we established a metabonomic method to evaluate metformin action in preventing obesity in a high fat diet-induced-obesity (DIO) mice model. 36 male C57BL/6 mice (8-week old) were randomly divided into control group ($n = 12$, normal chow), model group ($n = 12$, high fat chow) and metformin group ($n = 12$, high fat chow and dosed with metformin) over 16 weeks. A urinary metabonomic study using UPLC-TOF/MS was performed in combination with multivariate statistical analysis. In addition, indices of body weight and food intake as well as fasting blood glucose, fed blood glucose, oral glucose tolerance test (OGTT) and plasma insulin were collected. Significant weight loss in metformin-treated mice was achieved and 21 potential biomarkers were identified. Decreased glucose, myristic acid, stearidonic acid, lysoPC (16:0), lysoPC (18:0), L-glutamic acid, L-methionine, L-threonine, L-phenylalanine, L-histidine, L-carnitine, L-malic acid and pantothenic acid in urine indicated that metformin may have exerted effects on energy metabolism. Further, based on the biomarkers, we cautiously propose that tricarboxylic acid cycle (TCA) may have been compromised by metformin and might contribute to the activation of adenosine monophosphate kinase (AMPK), then AMPK activation led to more β-oxidation of certain fatty acids and augmented lipolysis and thus induced weight loss. Related cellular and molecular studies are being considered to further investigate the underlying mechanism.

1. Introduction

Obesity is increasing dramatically worldwide due to intake of diet with high energetic value and a sedentary lifestyle of modern society. Obesity is a metabolic disorder with a host of comorbidities, for instance, type 2 diabetes mellitus (T2DM), low-grade inflammation, cardiovascular diseases, hypertension and a number of malignances [1,2]. Thus, proper prevention and therapy strategies for obesity make sense not only for obesity itself, but also for the complexity between obesity and the comorbidities.

Metformin, an oral first line anti-diabetes agent, also had beneficial effects on cardiovascular system, polycystic ovary syndrome, anti-tumor action, etc. [3–6]. However, it is not difficult to discover that behind the wide therapeutic spectrum, metformin was intriguingly associated with weight loss in different disease subjects and animal models [3,7–9]. Therefore, weight loss of metformin action may be a point cut for in-depth evaluation of metformin. Yet, the majority of publications were basically about anti-diabetic action of metformin [7–13] and less available researches were focused on obesity. And publications have demonstrated that the mechanism

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of metformin action against T2DM was suppressing hepatic glucose production via AMPK activation [12,13], however, the relation between AMPK activation and weight loss induced by metformin has not yet been fully elucidated.

Metabonomics is a systemic platform for "global profiling" of endogenous metabolites of a certain physiopathological state in biofluids or tissues as a result of diseases, genetic modifications, exposure to a drug, toxin, environmental factors or other disturbances [2,14–19]. As metabonomics can provide holistic metabolic information of a certain physiopathological state, a metabonomic study of prevention treatment of metformin for obesity may bring forth new insights on the evaluation of metformin. Therefore, in this paper, a urinary UPLC-TOF/MS based metabonomic profiling with biochemical indices was employed to comprehensively evaluate metformin action in the prevention of obesity in a high-fat DIO mice model over a 16-week course. DIO model was established for it simulated modern lifestyle well.

2. Materials and methods

2.1. Reagents

Formic acid (HPLC grade) and leucine-enkephalin were purchased from Sigma–Aldrich (St. Louis, MO). Methanol and acetonitrile (HPLC grade) were obtained from Merck (Germany). Metformin and reference standards of L-lactate, choline, L-malate, N,N-dimethylglycine, calcium pantothenate, uric acid sodium salt, L-aspartic acid, L-phenylalanine, L-threonine, L-glutamic acid, L-histidine, L-methionine, L-carnitine, melatonin and creatine monohydrate were purchased from Sigma–Aldrich (St. Louis, MO). Reference standards of 1-hexadecanoyl-sn-glycero-3-phosphocholine (LysoPC (16:0) and 1-octadecanoyl-sn-glycero-3-phosphocholine (LysoPC (18:0)) were purchased from Avanti. Polar Lipids Inc. (Alabaster, AL, USA). Ultrapure water was prepared by a Milli-Q system (18.2 MΩ, Millipore, MA, USA). The normal chow and high fat chow (D12492) were purchased from Shanghai Laboratory Animal Co. Ltd. (Shanghai, China) and Research Diets (New Brunswick, NJ, USA), respectively.

2.2. Sample collection and preparation

36 male C57BL/6 mice (8-week old, with a mean body weight of 23.9 g) were purchased from Shanghai Laboratory Animal Co. Ltd. (Shanghai, China). Animals were raised in humidity and temperature controlled SPF room (humidity: 60%, temperature: 22 ± 3 °C), with a 12-h light and dark cycle and animals had free access to food and sterile water. Animal facilities and protocols were approved by the Animal Ethics Committee, Shanghai University of Traditional Chinese Medicine (Shanghai, China).

After 1-week acclimatization, mice were randomly divided into three groups (12 animals per group) as follows: control group, model group and metformin-treated group. Metformin-treated group was on a high fat diet and administered with metformin at a dosage of 250 mg/kg by oral gavage. Control group was fed with normal chow and model group was given high fat chow and these two untreated groups received the same volume of normal saline by gavage. Animals were all dosed daily between 9 a.m. and 10 a.m. Urine samples were collected at the end of 16 weeks and stored in −80 °C before analysis.

Thawed urine sample was centrifuged at 3500 rpm for 10 min at 4 °C, then 100 μL supernatant was obtained and 200 μL methanol was added to the supernatant and vortex-mixed for 2 min, then the mixture was centrifuged at 10,000 rpm for 15 min at 4 °C and 20 μL of the supernatant was transferred into injection vials and kept at −20 °C for UPLC TOF/MS analysis.

2.3. Biochemical measurements for blood glucose, OGTT and plasma insulin

Baselines of body weight and fed blood glucose were obtained before treatment from all the animals. Body weight and food intake were recorded every week. 2 h fed blood glucose was measured every 2 weeks and fasting blood glucose were measured on day 49, 70, 84 and 98 after fasting for 6 hours. 20% D-glucose saline solution (2 g/kg) was administered to perform OGTT on day 80 and 112 and plasma insulin was measured during OGTT. Blood glucose values were all determined by an ACCU-CHECK Active™ glucometer (Roche, Germany) and blood samples were obtained from the tail clip of animals.

2.4. UPLC-TOF MS analysis

Chromatographic separations were performed on an ACQUITY™ UPLC System (Waters Corporation, Milford, MA), equipped with a binary solvent delivery system and an autosampler. A BEH C18 column (2.1 mm × 100 mm, 1.7 μm) (Waters Corporation, Milford, USA) was used. The column was maintained at 40 °C with a flow rate of 0.4 mL/min. The mobile phase was composed of water (Phase A) and acetonitrile (Phase B) (containing 0.1% formic acid) and the optimized gradient elution program was set as follows: 0–3 min: 1–10% B; 3–7 min: 10–40% B; 7–8.5 min: 40–100% B; 8.5–10 min: 100% B; 10–10.5 min: 100–99% B; 10.5–12.2 min: 1% B.

MS spectrometry was carried out on a Waters Q-TOF Premier MS system (Waters Corp., Milford, MA) with an electrospray ionization source (ESI) operating in positive ion mode. Nitrogen was used as the drying gas .The desolvation gas flow rate was 750 L/h and desolvation temperature was maintained at 350 °C. Cone gas flow rate was maintained at 40 L/h and the source temperature was set at 120 °C. Capillary voltage and cone voltage were 2800 V and 40 V, respectively. The scan time and interscan delay were 0.28 s and 0.1 s, respectively. All analyses were obtained using an independent reference lock mass ion to ensure accuracy and reproducibility and leucine-enkephalin (m/z 566.2771 in positive mode) was used as the lock mass at the concentration of 50 pg/μL and a flow rate of 10 mL/min. Data was acquired in centroid mode with a scan range from 50 to 1000 and a lockspray frequency of 10 s and averaged over 10 scans for correction.

2.5. Data collection and analysis

UPLC/MS spectra data were first processed by Markerlynx Applications Manager Version 4.1 (Waters, Manchester, UK), including the detection and retention time (R.T.) alignment of peaks in each chromatogram by Apex-Track-peak detection package incorporated in this software. The data were combined into a single matrix after aligning peaks with retention time-exact m/z pair and associated peak intensity. Then, ion intensities of each detected peak were normalized within each sample, to the sum of the intensities in that sample. Specifically, some parameters proposed by Cong et al. [18] were used as parameters for data processing and were set as follows: retention time: 0–12.30 min, mass range: 50–1000 Da, mass tolerance: 0.05 Da, minimum intensity: 15% of the base peak intensity, maximum mass per retention time: 6 and retention time tolerance: 0.04 min. The processed data were then introduced to SIMCA-P version 11.5 (Umetrics, Umea, Sweden). Multivariate statistical analysis of the data: Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA) were performed. PCA is an unsupervised multivariate statistical analysis method that transforms correlated variables of a dataset into a smaller number of independent variables, i.e., the principal components. PCA model was used as an overview to see the outliers,

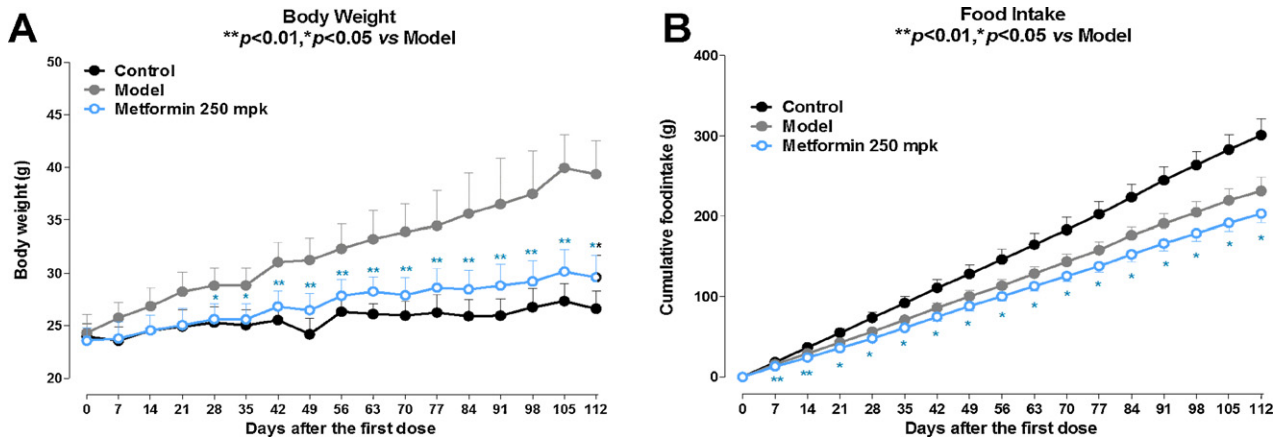

Fig. 1. Body weight (A) and food intake (B) of mice during 0–16 weeks.

groups and trend of data and PLS-DA was then constructed to bring out specific variations between different treatment groups. Before further interpretation, a 7-round cross-validation and a 999 random permutation test (*Y* Scramble) was also employed to avoid overfitting. The idea of this validation is to compare the goodness of fit (Q^2 and R^2) of the original model with the goodness of fit of several models based on the data where the order of *y*-observations has randomly permutated while the *x*-matrix has been kept intact. Parameters R^2Y and Q^2Y are indicators of the model's fitness and predictability and the model is considered excellent when both R^2Y and Q^2Y are near to 1 and considered a reliable model when $Q^2Y \geqq 0.4$ [19]. Variable Importance in the Projection (VIP) value of validated PLS-DA model is taken as the measurement index for peak selecting, meaning higher VIP value stands for greater contribution of a variable to the separation of different treatment groups and variables with VIP >1.0 are supposed to contribute significantly

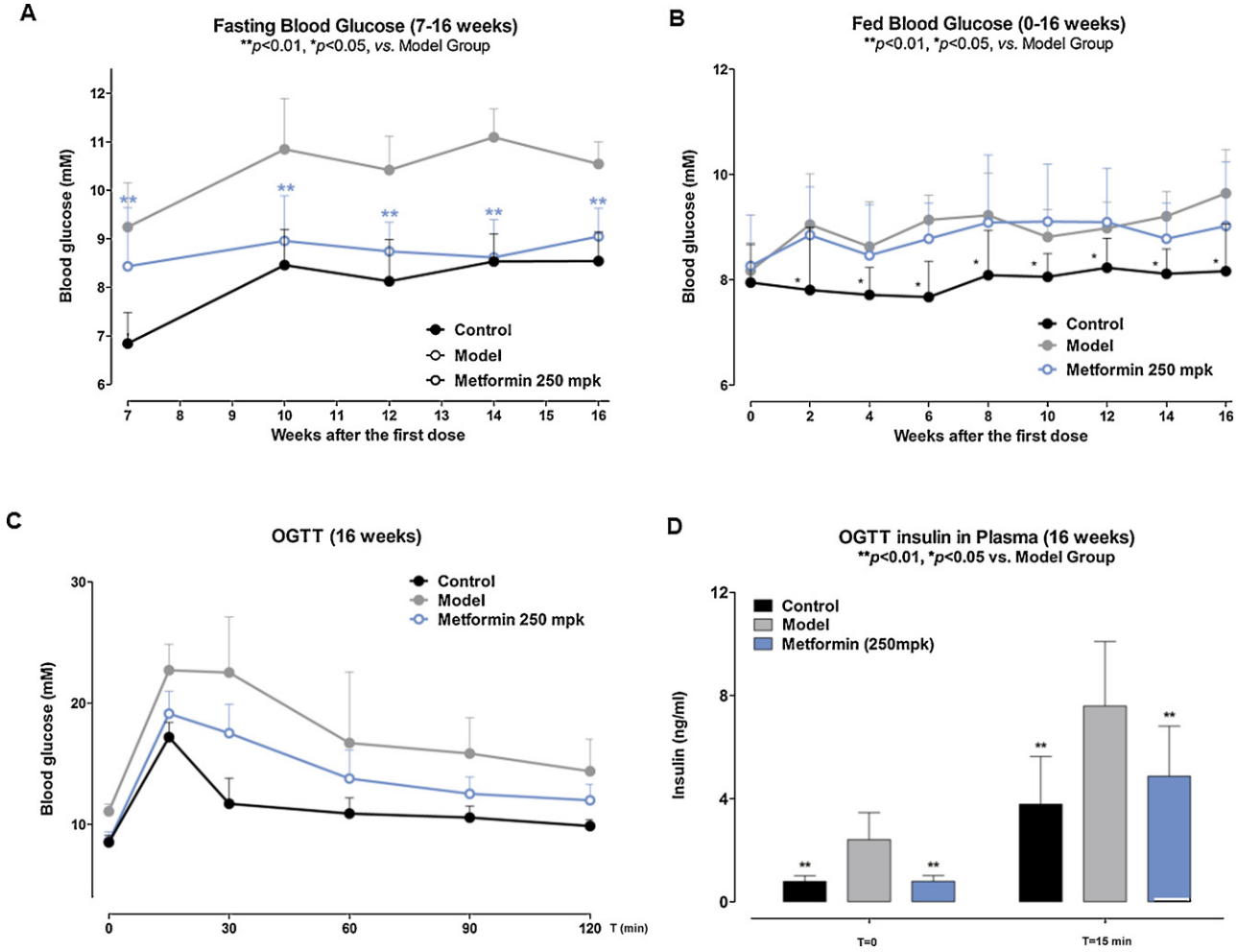

Fig. 2. Fed blood glucose during 0–16 weeks (A) and fasting blood glucose during 7–16 weeks (B), OGTT (C) and plasma insulin (D) performed on 16 weeks.

to the separation. Student's *t*-test was also performed to select significantly differential peaks (*p*-value <0.05) according to their intensities.

3. Results

3.1. Effect of metformin on general and clinical biochemical indices of animals

Compared with controls, body weight in model group increased by 61.8% after 16 weeks and showed elevated fed blood glucose (9.05 ± 0.97 vs. 7.81 ± 1.19 mM, model group vs. control group, *p*-value <0.05) after 2 weeks and fasting blood glucose increased significantly (9.24 ± 0.91 vs. 6.84 ± 0.64 mM, model group vs. control group, *p*-value <0.01) after 7 weeks. Animals in model group developed impaired glucose tolerance and hyperinsulinemia, and insulin resistance became overt after 7 weeks, indicating a high-fat DIO model with prediabetic characteristics (impaired fasting glucose and impaired glucose tolerance) was successfully established. Body weights of control, model and metformin group at baseline are 23.95 ± 1.22 g, 24.31 ± 1.72 g and 23.58 ± 1.19 g respectively. Body weight (0–16 weeks) and food intake (0–16 weeks) of the three groups are presented in Fig. 1, fed blood glucose of control, model and metformin group at baseline is 7.95 ± 0.72 mM, 8.18 ± 0.53 mM and 8.27 ± 0.97 mM. Fed blood glucose (0–16 weeks), fasting blood glucose (7–16 weeks), OGTT and plasma insulin (16 weeks) are presented in Fig. 2.

Metformin exerted an inhibitory effect on animal food intake and body weight gain. After 16 weeks, animals in model group reached an average body weight of 39.35 g and an accumulative food intake of 231.2 g, whereas animals in metformin-treated group maintained at 26.62 g (*p*-value <0.01) and 202.9 g (*p*-value <0.05), respectively. Compared with model group, metformin remarkably lowered fasting blood glucose over the whole treatment (*p*-value <0.01), while had little effect on fed blood glucose. In addition, metformin treatment led to decreased plasma insulin and improved glucose tolerance, demonstrating the prevention efficacy of metformin against obesity and obesity-related metabolic disorders.

3.2. UPLC-TOF MS method validation

In our preliminary study, methanol was added to urine samples to avoid protein "plug" phenomenon and is a conventional extraction method in metabonomic studies [18]. The volume ratio of methanol added to urine sample: 1:1, 2:1 and 3:1 was investigated before the full experiment. As chromatogram of the volume ratio of 2:1 produced more detectable peaks and stronger peak intensities with lower noise, matrix effect and base line, the ratio of 2:1 was chosen for sample preparation. Full scan of urinary metabolites was set in positive mode for more information was obtained than in negative mode. The optimization of mobile phase, flow rate and gradient elution program and mass parameters (desolvation gas flow rate and temperature, cone gas flow rate, source temperature, capillary voltage and cone voltage) were all investigated to guarantee lower base line and noise-to-signal ration, less analytical time and more information-richness. 0.1% formic acid was added to the mobile phase to suppress peak tailing and enhance high separation efficacy. A representative base peak intensity (BPI) chromatogram in positive mode is presented in Fig. 3.

Method validation was carried out using 10 ions of chromatographic peaks. Precision and reproductivity of UPLC-TOF MS technique were validated by 6 replicated analyses of one sample and 6 parallel samples prepared by the same protocols (data not shown). The relative standard deviations of peak intensities and areas of the 10 ions were <0.5%, demonstrating that the established method was valid.

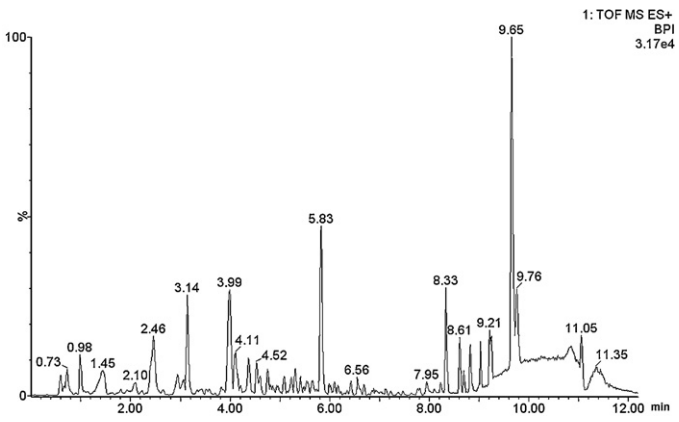

Fig. 3. Representative base peak intensity chromatogram of mice urine obtained in electrospray ionization positive mode based on UPLC-TOF MS.

3.3. Effect of metformin on urine metabolic profiling

The PLS-DA model (Fig. 4) ($R^2X = 0.442$, $R^2Y = 0.954$, $Q^2 = 0.933$) was constructed to visualize the influence of metformin on metabolic profiling of different treatment groups and identify potential biomarkers. As is seen, clear separation of three different treatment groups was achieved, suggesting biochemical changes happened in the urine of model and metformin-treated group. Furthermore, R^2Y-intercept and Q^2Y-intercept of the permutation test were 0.198 and −0.222, respectively. The criteria for validity of a certain model are: all blue Q^2 values in the left are lower than the original points in the right; the blue regression line of Q^2 points intersects the vertical axis (on the left) at or below zero. Therefore, the 7-round cross-validation and 999 random permutation test (Fig. 5) showed good predictability and no overfitting of the PLS-DA model. Differential metabolites which were accountable for intergroup variation with VIP value >1.0 and Student's *t*-test *p*-value <0.05 are listed in Table 1 (the significance level (*p*-value) was set at 0.05). 21 metabolites were chosen as potential biomarkers and among them, 18 metabolites were validated by reference standards and identification of the other metabolites were obtained by searching databases of HMDB (http://www.hmdb.ca) and KEGG (http://www.genome.jp). Compared with normal controls, glucose, L-lactic acid, L-glutamic acid, L-threonine, L-phenylalanine, myristic acid, stearidonic acid and uric acid increased, while L-malic

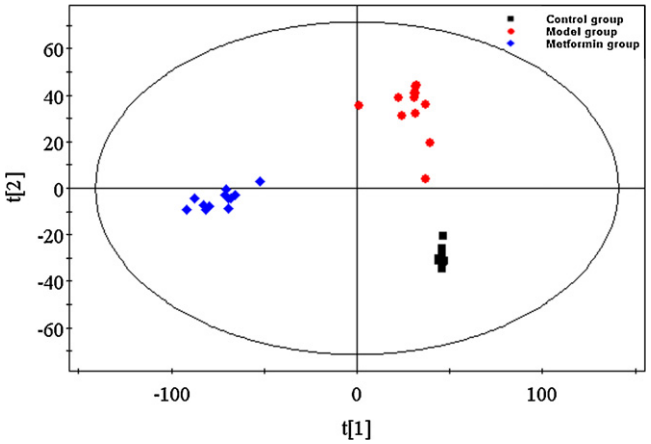

Fig. 4. PLS-DA score plot of mice urine of different groups from UPLC-TOF MS (*n* = 12).

Table 1
Identification of significantly differential metabolites in the mice urine.

R.T. (min)	*m/z* (Da)	Selected ion	Elemental composition	Identification results	Metabolic pathway	Model[b]	Metformin[b]
4.66	181.0856	$[M+H]^+$	$C_4H_6O_4$	Glucose[a]	Glucose metabolism	(+)↑	(−)↑
4.59	91.0543	$[M+H]^+$	$C_3H_6O_3$	L-Lactic acid[a]	Glycolysis	(+)↑	(+)↓
1.00	135.0321	$[M+H]^+$	$C_4H_6O_5$	L-Malic acid[a]	TCA cycle	(+)↓	(+)↓
2.40	220.1173	$[M+H]^+$	$C_9H_{17}NO_5$	Pantothenic acid[a]	CoA biosynthesis	(+)↓	(+)↓
0.72	148.1329	$[M+H]^+$	$C_5H_9NO_4$	L-Glutamic acid[a]	Glutamate metabolism	(+)↑	(+)↓
4.37	88.0221	$[M-COOH]^+$	$C_4H_7NO_4$	L-Aspartic acid[a]	Aspartic acid metabolism	(+)↓	(+)↓
4.27	175.0807	$[M+H]^+$	$C_6H_{14}N_4O_2$	L-Arginine	Arginine and proline metabolism	(+)↑	(+)↓
2.64	120.0444	$[M+H]^+$	$C_4H_9NO_3$	L-Threonine[a]	Glycine, serine and threonine metabolism	(+)↑	(+)↓
0.99	150.0584	$[M+H]^+$	$C_5H_{11}NO_2S$	L-Methionine[a]	Cysteine and methionine metabolism	(+)↓	(+)↓
0.98	166.0722	$[M+H]^+$	$C_9H_{11}NO_2$	L-Phenylalanine[a]	Phenylalanine and tyrosine metabolism	(+)↑	(+)↑
0.67	156.0421	$[M+H]^+$	$C_6H_9N_3O_2$	L-Histidine[a]	Histidine metabolism	(+)↓	(+)↓
5.82	103.0543	$[M+H]^+$	$C_4H_6O_3$	Acetoacetic acid	Fatty acid metabolism	(+)↓	(+)↓
0.70	162.1126	$[M+H]^+$	$C_7H_{15}NO_3$	L-Carnitine[a]	Fatty acid metabolism	(+)↓	(+)↓
9.09	204.1385	$[M+H]^+$	$C_9H_{17}NO_4$	L-Acetyl carnitine	Fatty acid metabolism	(+)↓	(+)↓
7.99	229.1256	$[M+H]^+$	$C_{14}H_{28}O_2$	Myristic acid	Fatty acid metabolism	(+)↑	(+)↓
4.67	277.1306	$[M+H]^+$	$C_{18}H_{28}O_2$	Stearidonic acid	Fatty acid metabolism	(+)↑	(−)↓
10.19	413.2669	$[M+H]^+$	$C_{18}H_{39}NO_7P$	LysoPC (10:0)	Lipid metabolism	(+)↓	(+)↓
9.32	482.3605	$[M+H]^+$	$C_{23}H_{48}NO_7P$	LysoPC (15:0)	Lipid metabolism	(+)↑	(−)↓
9.20	496.3405	$[M+H]^+$	$C_{24}H_{50}NO_7P$	LysoPC (16:0)[a]	Lipid metabolism	(+)↑	(−)↓
9.62	524.3734	$[M+H]^+$	$C_{26}H_{54}NO_7P$	LysoPC (18:0)[a]	Lipid metabolism	(+)↑	(−)↓
7.96	568.2966	$[M+H]^+$	$C_{30}H_{50}NO_7P$	LysoPC (22:6)	Lipid metabolism	(+)↓	(+)↓
9.14	429.2973	$[M+H]^+$	$C_{29}H_{48}O_2$	Cholesteryl acetate	Lipid metabolism	(+)↓	(+)↓
0.71	132.0797	$[M+H]^+$	$C_4H_9N_3O_2$	Creatine[a]	Arginine and proline metabolism	(+)↓	(+)↓
0.97	169.0537	$[M+H]^+$	$C_5H_4N_4O_3$	Uric acid[a]	Purine metabolism	(−)↑	(+)↓
6.16	177.1273	$[M+H]^+$	$C_{10}H_{12}N_2O$	Serotonin	Tryptophan metabolism	(+)↓	(+)↓
0.95	209.0556	$[M+H]^+$	$C_{10}H_{12}N_2O_3$	L-Kynurenine	Tryptophan metabolism	(+)↑	(+)↓
0.69	105.1114	$[M+H]^+$	$C_5H_{14}NO$	Choline[a]	Choline metabolism	(+)↓	(+)↓
0.68	104.1067	$[M+H]^+$	$C_4H_9NO_2$	N,N-dimethylglycine[a]	Choline metabolism	(+)↓	(+)↓
5.07	233.0918	$[M+H]^+$	$C_{13}H_{16}N_2O_2$	Melatonin[a]	Others	(+)↓	(+)↓
4.90	165.0913	$[M+H]^+$	$C_9H_8O_3$	Phenylpyruvic acid	Others	(+)↓	(−)↓

All data represented the peak intensity values of urinary metabolites on day 112. The upward arrow (↑) means elevated level and the downward arrow (↓) means lowered level of the metabolite.

[a] Compounds confirmed by reference standards.

[b] Compared to the controls, "+" means statistical significant difference and "−" means no statistical significant difference. The significance level (*p*-value) was set at 0.05.

acid, L-carnitine, choline, N,N-dimethylglycine, pantothenic acid, L-methionine, L-aspartic acid, L-histidine, melatonin and creatine decreased in model group. On the whole, after metformin treatment, these metabolites had a tendency to decline in comparison with the model group. Of note, glucose, myristic acid, stearidonic acid, L-glutamic acid, L-threonine, L-phenylalanine, LysoPC (15:0), LysoPC (16:0), LysoPC (18:0), L-lactic acid and uric acid were reversed to normal levels by metformin to certain degrees. The fold change (FC) and *p*-value of the 21 potential biomarkers based on peak intensity value are listed in Table 2 and extracted ion chromatograms of standards of 15 potential biomarkers are shown in Figs. 6 and 7.

Table 2
Fold change (FC) and *p*-value of Student's *t*-test of potential biomarkers in urine based on peak intensity values.

No.	Metabolites	Model vs. control		Metformin vs. control	
		FC	*p*-Value[a]	FC	*p*-Value[a]
1	Glucose	7.62	4.95E−8	1.45	–
2	L-Lactic acid	1.23	0.013	0.44	1.82E−10
3	L-Malic acid	0.28	1.49E−3	0.01	1.03E−4
4	L-Carnitine	0.27	5.89E−7	0.02	7.46E−8
5	Choline	0.60	0.032	0.15	9.25E−5
6	N,N-dimethylglycine	0.58	0.017	0.15	3.69E−5
7	Pantothenic acid	0.42	5.19E−7	0.02	2.97E−8
8	L-Glutamic acid	1.70	2.56E−3	0.08	1.73E−4
9	L-Threonine	1.34	0.014	0.40	8.13E−9
10	L-Methionine	0.37	3.84E−5	0.06	1.33E−6
11	L-Phenylalanine	2.20	3.73E−4	1.28	0.012
12	L-Aspartic acid	0.46	8.16E−4	0.09	4.17E−6
13	L-Histidine	0.42	7.18E−4	0.05	1.32E−5
14	Myristic acid	6.61	1.11E−6	2.93	2.55E−6
15	Stearidonic acid	7.34	2.21E−7	0.42	–
16	LysoPC (15:0)	2.16	4.51E−4	0.88	–
17	LysoPC (16:0)	1.63	0.031	0.04	2.02E−4
18	LysoPC (18:0)	5.54	4.30E−4	0.32	–
19	Melatonin	0.79	0.045	0.07	9.39E−8
20	Creatine	0.48	5.14E−6	0.07	6.12E−9
21	Uric acid	1.52	–	0.04	1.48E−4

[a] Compared to the controls, "−" means no statistical significant difference.

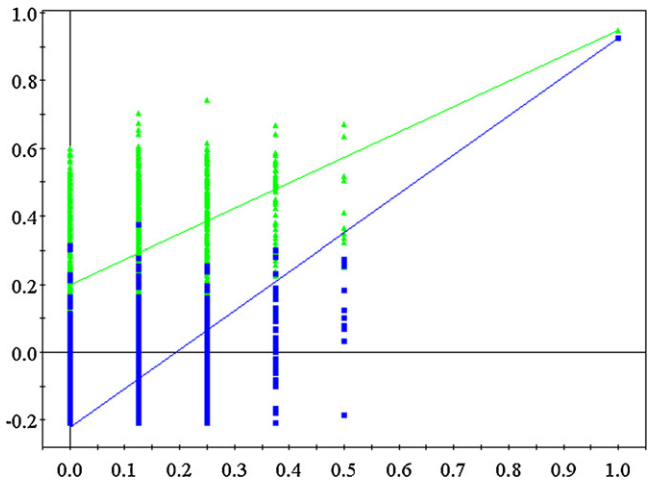

Fig. 5. The permutation test ($n = 999$ times) of the PLS-DA model. ((▲) represented R^2 and (■) represented Q^2, the x-axis means the correlation coefficient between the original y variable and the permutated y variable and the y-axis is the value of R^2 and Q^2).

4. Discussion

Obesity prevention is widely acknowledged crucially important and researches have demonstrated that prevention strategies including intensive lifestyle modification and pharmaceutical agent interventions were effective with different efficacy [8]. We established a high-fat DIO mice model to best simulate obesity in human population to assess metformin action in preventing obesity based on a metabonomic platform. In our study, metformin treatment remarkably inhibited weight gain and food intake and reduced food intake may partially contribute to the weight loss of metformin treatment. Further, metabonomic results shed some new light on metformin action against obesity.

TCA cycle is the major source of ATP production in bio-organism and acetyl Coenzyme A (CoA) is an intermediary metabolite in TCA cycle. Pantothenic acid is a constituent part of acetyl CoA [20,21], thus, variation of pantothenic acid is supposed to exert influence on acetyl CoA metabolism, which consequently affects TCA cycle. Herein, we found a marked drop of pantothenic acid in metformin group compared with the obese ones. Interestingly, malic acid, another intermediate in TCA cycle also decreased in metformin-treated mice. Diminution of pantothenic acid and malic acid implied that TCA activity might have been compromised by metformin. Once TCA activity is suppressed, we assume that cellular adenosine triphosphate/adenosine monophosphate (ATP/AMP) ratio will decrease, which would facilitate AMPK activation. As a result, certain cellular catabolism would be switched on and anabolic pathways switched off, in order to provide for more ATP [22,23]. As L-carnitine assists long chain fatty acids to go into mitochondrial matrix to be oxidized, reduced L-carnitine in metformin group was indicative that the β-oxidation of certain fatty acids may have been augmented and hence resulting in a reduction of myristic acid and stearidonic acid, suggesting that more lipolysis was induced by metformin through AMPK activation and may there-

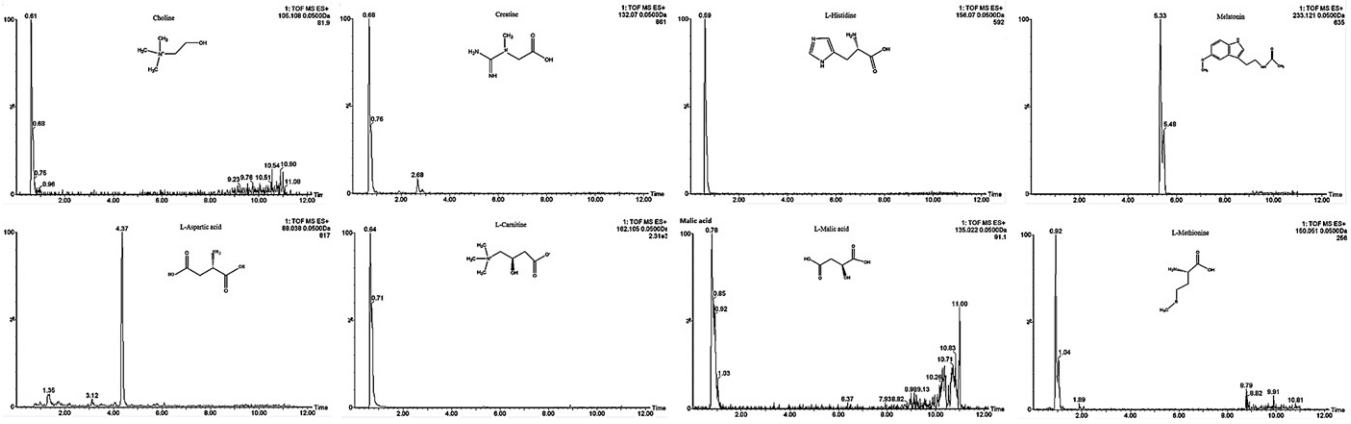

Fig. 6. Extracted ion chromatograms of standards of choline, creatine, L-histidine, melatonin, L-aspartic acid, L-carnitine, L-malic acid and L-methionine.

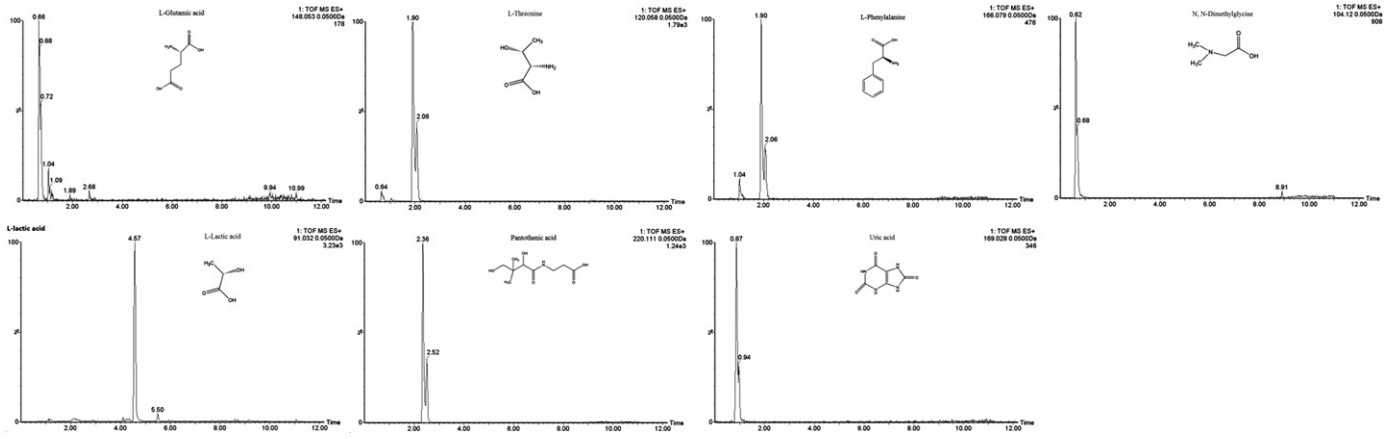

Fig. 7. Extracted ion chromatograms of standards of L-glutamic acid, L-threonine, L-phenylalanine, N,N-dimethylglycine, L-lactic acid, pantothenic acid and uric acid.

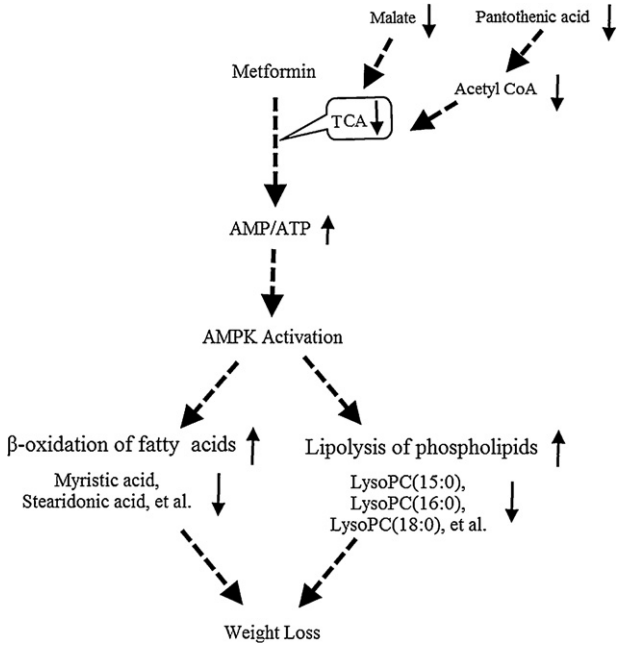

Fig. 8. Possible mechanism of metformin inducing weight loss.

fore contribute to weight loss. To date, lysophosphatidylcholines (Lyso PCs) have been reported to be the biomarkers of obesity, T2DM, metformin action and a number of cancers [24–27]. We found elevated levels of LysoPC (15:0), LysoPC (16:0) and LysoPC (18:0) in model group and metformin reduced these metabolites to certain degrees. As LysoPCs can also serve as a source of energy, reduction of LysoPCs could be attributed to the enhanced catabolism of phospholipids caused by the activation of AMPK and therefore, contributed to weight loss by metformin treatment. In addition, compared with the model group, lowered level of glucose and amino acids (see Table 1) could also be interpreted as the down-regulated synthesis and/or up-regulated catabolism of carbohydrates through activation of AMPK by metformin. To sum up, metformin may have suppressed TCA cycle which might partly contributed to AMPK activation, resulting in the up-regulated β-oxidation of certain fatty acids, lipolysis of lipids and phospholipids and catabolism of amino acids and glucose, therefore, induced weight loss and lowered blood and urinary glucose (see Fig. 8).

5. Conclusion

Urinary metabonomic findings together with clinical indices revealed that long-term treatment of metformin could effectively prevent C57BL/6 mice from developing obesity. Metformin may inhibit weight accumulation via activating AMPK partly through suppressing TCA cycle, as reduced pantothenic acid and malic acid were observed in metformin-treated mice. As a result, up-regulated β-oxidation of certain fatty acids, lipolysis of lipids and phospholipids might contribute to weight loss induced by metformin. Additionally, catabolism of amino acids and glucose was also enhanced by metformin.

Conflict of interest

None declared.

Acknowledgments

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