Alterations of Metabolic Profile in The Plasma of High-fat High-fructose Diet-induced Obese Rats

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Abstract. Obesity, a kind of metabolic diseases, is tightly linked to many chronic diseases, such as cardiovascular diseases, non-alcohol fatty liver diseases and diabetes. However, the underlying molecular mechanisms of obesity remain uncertain. In order to comparatively analyze the plasma metabolome and present some clues for relative research on recovering the underlying molecular mechanisms, we performed a gas chromatography–mass spectrometry-based metabolic profiling of the plasma of obese rats induced by high-fat high-fructose diet. The changes of plasma metabolism between the diet-induced obese rats (n=8) and healthy control rats (n=7) were studied and the significant metabolites were identified by multivariate statistical analysis, including principal component analysis (PCA) and pair-wise orthogonal projections to latent structures discriminant analysis (OPLS-DA). Compared with control rats, diet-induced obese rats were characterized by 16 lower level metabolites and 23 higher level metabolites. These molecular changes were closely related to amino acid metabolism, carbohydrates metabolism and lipid metabolism. These findings may provide insight into the pathophysiological mechanisms underlying obesity and could be of valuable assistance in the early clinical diagnosis of obesity associated diseases.

1. Introduction
As a kind of metabolic disease, obesity has aroused extensive interest among researchers. Obesity is closely associated with the daily lifestyle and diet custom, most of which are caused by high-fat and high-sugar diets. Increasing evidences support the view that obesity is a risk factor for cardiovascular diseases [1, 2], diabetes [3], and cancers [4, 5]. Haemmerle et al. showed that disruptions of the dynamic equilibrium of lipid synthesis and lipid catabolism underlaid metabolic diseases such as obesity and type II diabetes [6]. Metabolites are transformed to the chemical entities during metabolism, and provide a functional readout of cellular biochemistry [7]. And these metabolites are mainly transported through the blood. Thus, we decided to investigate the metabolites of plasma of obese rats to find some useful information to conduct the obese research.

Metabolomics is a rapidly developing branch in the field of system biology. It is mainly concerned with the analysis of all small molecules or metabolites in the body or specific regions of the body, so as to provide tools for the study of metabolic changes caused by the changes of disease-related genes and protein [8, 9].
Therefore, we used the plasma of obese rats induced by high-fat and high-fructose diets to study the profile of the metabolic spectrum of obesity. This study can elucidate the changes of metabolism in obesity and provide a reference for the study of the potential mechanism of obesity.

2. Materials and methods

2.1 Animals

Fifteen healthy male Sprague-Dawley (SD) rats aged 5 weeks were provided by the Guangdong medical laboratory animal center. The rats adapted after a week and were randomly divided into two groups: the control group (Cont, n=7) and the model group (HFF, n=8). Rats had a 12-hour light/dark cycle, 60% humidity, 18-22°C temperature and provided ad libitum access to assigned diet and water. Additionally, HFF groups had continuous access to a separate bottle with HFCS-55 (HFCS-55: 55% fructose, 45% glucose diluted with water to 12.5% solution). Custom rodent diets were purchased from the Guangdong medical laboratory animal center, with 10% or 60% total kcal from fat. The control group were fed a standard diets (No: D12450-B), and the model group were received a high fat diets (No: D12492). The weight of rats was measured at 8th week and 15th week. All of the studies were performed with the approval of the experimental animal committee of Guangdong pharmaceutical university, Guangdong, China. After the rats were deep anesthetized, blood samples from abdominal aorta was collected, centrifuged and stored at -80°C. After the rats were sacrificed, the epididymal white adipose tissue (EpWAT) was removed and weighted.

2.2 Non-targeted metabolomics analysis

2.2.1 Sample preparation

Sample preparation was carried out as described Li et al.[10] with slight modification. Briefly, 50µL of rat's plasma was mixed with 10µL of nonadecanoic acid methanol solution (1mg/mL, w/v) and 250µL of H₂O-MeOH-CHCl₃ solution (2:5:2, v/v/v), then stayed at 4°C for 20min. The mixture was next centrifuged at 14,000rpm for 15min at room temperature. 200µL of supernatant was taken and dried in nitrogen, which was followed by mixed with 80µL of methoxyamine pyridine solution (15mg/mL, w/v) and incubated at 37°C for 90min. Finally, 80µL of bis-(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% chlorotrimethyl-silane (TMCS) was added and incubated at 70°C for 1h. Nonadecanoic acid was used as internal standard substance.

2.2.2 GC–MS analysis

The GC-MS analysis was employed by designed as Li et al.[10] with slight modification. The derivatized samples were analyzed using Agilent 7890B GC system (Agilent Technologies, Santa Clara, CA, USA) with a splitting model. Separation was carried out on an HP-5 MS fused silica capillary column (60 m x 0.25 mm x 0.25 m, Agilent Technologies), with high-purity helium as the carrier gas at a constant flow rate of 1.0 ml/min. The GC temperature programming was set to begin at 60°C for 1 min and then increased from 60 to 100°C at 8°C /min, from 100 to 170°C at 15°C /min, from 170 to 210°C at 10°C /min, and from 210 to 350°C at 15°C /min, with a final 2 min maintenance at 350°C. The column effluent was introduced into the ion source of an Agilent 5977B mass selective detector (Agilent Technologies). The temperature of injection and ion source was set to 260°C and 230°C, respectively. Electron impact ionization (~70 eV) was used. MS detection was conducted with electron impact ionization mode in the full scan mode from mass (m/z) 50 to 500.

2.2.3 Identification of metabolic signatures

GC–MS metabolic profiles were processed by Masshunter workstation B.07.00 equipped with the Database National Institute of Standards and Technology (NIST) 14.L. The Qualitative Analysis and Quantitative Analysis were applied respectively to identify and quantify the compounds. The results were importing into SIMCA-P 14.1 (Umetrics, Umeå, Sweden) for statistical analysis. Principal
component analysis (PCA) and pair-wise orthogonal projections to latent structures discriminant analysis (OPLS-DA), were implemented to identify differential plasma metabolites between diet-induced obese rats and healthy controls. The discriminating metabolites were judged by variable influence on projection values (VIP) from the OPLS-DA model (VIP > 1.0) and two-tailed Student’s t-test ($p < 0.05$). Then, all the differential metabolites was classified based on the Human Metabolome Database (http://www.hmdb.ca/).

2.2.4 Metabolic pathway and function analyses
MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/), which helps to comprehensive metabolomics data analysis and visualization [11], was adapted. These significantly changed metabolites were performed pathway analysis to evaluate the most significantly altered metabolic pathways. And possible biological roles through the enrichment and pathway analysis function of MetaboAnalyst 4.0.

2.3 Statistical analysis
All values were expressed as the mean ± SD. Significance was set at an alpha level of 0.05. A statistical package SPSS22.0 (SPSS Inc., Chicago, IL, USA) was used for analyses. Graphpad Prism 6.0 software (GraphPad, CA, USA) was used for graphics.

3. Results

3.1 The effect of high fat high fructose diet on the rats
Significant differences in body weight began to occur between the control group and the high-fat and high-fructose group (HFF) after feeding for about 8 weeks (Figure 1.A). Figure 1 illustrated the diet-induced rat obesity model was constructed.

Figure 1. The effect of diet on control and model groups. (A) Change of body weight in the control and model group. (B) The weight of epididymal white adipose tissue (EpWAT) in two groups of rats on different diets. *$p < 0.05$, **$p < 0.01$.

3.2 Metabolomic analysis
Representative GC–MS total ion current chromatograms from both groups are shown as Figure 2. All the peaks show strong signal, high peak capacity and good reproducibility. Referring to the NIST database, about 215 compounds were detected in the plasma samples of each group of rats after the internal standard was removed.
Figure 2. Representative GC-MS total ion chromatograms of the control and diet-induced groups. (A) Cont group; (B) HFF group.

To verify whether the high-fat and high-fructose diet had an effect on the metabolism of SD rats, we used PCA analysis to deal with the GC-MS metabolic profiles recorded as abovementioned. The PCA scores plot suggested that the clear differences exist between the Cont and HFF groups. The data showed that the diet-induced rats were obviously discriminated from the control groups ($R^2_X = 0.526$), and diet did change the metabolic pattern of SD rats (Figure 3A, 3B). OPLS-DA analysis was applied to maximize the discrimination between the two groups. The OPLS-DA loading plots revealed that the model was efficient, and there was a significant separation between the HFF group and the Cont group (Figure 3C). Moreover, permutation testing also showed that the OPLS-DA model was reliable, as the original $R^2$ and $Q^2$ values to the right were significantly higher than the corresponding permuted values to the left (Figure 3D).

3.3 Identification of significant metabolites

Based on a threshold of variable influence on projection values (VIP) from the OPLS-DA model (VIP > 1.0) and two-tailed Student’s t-test ($p < 0.05$), forty-one differential metabolites in the HFF and Cont groups were screened. Compared with the control groups, 16 metabolites were decreased and 23 metabolites were increased in model groups. The HMDB was used for classification of the 39 metabolites. These metabolites were sub-grouped as Amino acids, peptides, and analogues (26%), carbohydrates and carbohydrate conjugates (23%), fatty acids and conjugates (13%), dicarboxylic acids and derivatives (8%), and alpha hydroxy acids and derivatives (8%) (Figure 4A). These metabolites were mainly located in extracellular (37%), cytoplasm (32%), mitochondria (14%) membrane (8%) and lysosome (3%) cellular locations (Figure 4B).
Figure 3. Multivariate statistical analysis of GC-MS spectra of plasma of the control and model groups. (A) Principal component analysis (PCA) score plot; (B) Hotelling’s plot of principal component analysis; (C) Pair-wise orthogonal projections to latent structures discriminant (OPLS-DA) scores plot; (D) Statistical validation of the OPLS-DA model by permutation testing.

Figure 4. Pie chart of the classification of potential metabolic disturbances in diet-induced obese rats compared with control rats. Representation of metabolites of (A) chemical taxonomy, (B) cellular locations.

3.4 Metabolic pathway and function analyses
Pathway and enrichment analysis showed that the differential metabolites identified were primarily associated with amino acid metabolism, energy metabolism and lipid metabolism. The detail results of pathway and function analysis were shown in Figure 5.
Figure 5. Summary of enrichment and pathway analysis with MetaboAnalyst. (A) the enrichment analysis of metabolites. (B) the pathway analysis of metabolites. (a) D-glutamine and D-glutamate metabolism, (b) alanine, aspartate and glutamate metabolism, (c) glyoxylate and dicarboxylate metabolism, (d) valine, leucine and isoleucine biosynthesis, (e) arginine and proline metabolism

4. Discussion
According to the Figure 1, there was a significant difference in body weight between the two groups up to 8th week. It indicated that the model group could be obese. Lipid accumulation in white adipose tissue (WAT) and the weight of three types of fat pad mass play a key role in the increase in body weight in diet-induced obese rats [12]. Epididymal adipose tissue is generally considered to be pure WAT with a characteristic structure and function [12]. To further confirm that weight gain is not due to muscle gain, we measured the weight of epididymal adipose tissue at 15th week. The results showed that diet induced obesity rat model successfully.
Metabolomic, which analyzes small molecule metabolites of an organism or a specific biological sample, provides an insight into the discovery of biomarkers and therapeutic targets in biomedicine [13]. To better understand the underlying pathogenesis of obesity, the GC-MS metabolomics approach was used. By employing the Non-targeted metabolomics analysis, our study revealed 39 metabolites clearly distinguishing HFF from Cont rats. And classification of metabolites, possible metabolic pathways as well as possible relevant genes and proteins of metabolites were also matched. The five most significantly different pathway are: (1) D-glutamine and D-glutamate metabolism, (2) alanine, aspartate and glutamate metabolism, (3) glyoxylate and dicarboxylate metabolism, (4) valine, leucine and isoleucine biosynthesis, (5) arginine and proline metabolism.

We found that the amino acid, including D-valine and L-glutamic acid, citrulline, methylmalonic acid and D-aspartic acid were significantly altered, which mainly took part in the valine, leucine and isoleucine metabolism and arginine and metabolism. Based on the pathway analysis, the arginine and proline metabolism was significant changed. Xia et al. reported that disrupted arginine and proline metabolism might contribute to the development of overweight and obesity in school-age children [14]. This research also has shown that plasma amide acid concentrations positively associated with β-cell function relative to insulin sensitivity [15]. In present research, significantly elevated glutamic acid was observed. Nagao et al. reported that high intracellular glutamate potentially related to adipocyte dysfunction [16]. These findings corroborate previous reports that suggest disturbed amid metabolism may be involved in the pathophysiology of obesity.

Compared with the control group, palmitic acid was increased in this study. Stephen C et al. reported palmitic acid mediates hypothalamic insulin resistance by altering PKC-θ subcellular localization in rodents [17]. Increasing evidence showed that increasing palmitic acid can cause inflammation and insulin resistance, by activating proinflammatory pathways [18-20] and impairing the function of the endoplasmic reticulum (ER) and mitochondria [21, 22]. Besides, an excess of palmitic acid lead to the development of nonalcoholic fatty liver disease (NAFLD) [23]. Therefore, these results implied that palmitic acid may contribute to the further research of obesity.

This research is to explore the potential biomarkers under the physiological obese state and the metabolism pathway with the strongest correlation through plasma metabolome, so that we can seek for small molecular metabolites that can medicate the physiological and pathological changes of obesity and complication stemming from obesity. But further research is still to be done.

5. Conclusions

In conclusion, 15 weeks of high-fat and high-fructose diet induced obesity successfully. GC-MS metabolic profiling and multivariate statistical analysis showed significant alteration in diet-induced obesity rats plasma metabolism. And the most altered pathways were amino acid metabolism, energy metabolism and lipid metabolism. The most obviously changed metabolites were L-glutamic acid, citrulline, methylmalonic acid, palmitic acid and L-aspartic. These data provide a reference for the further research of obesity.

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