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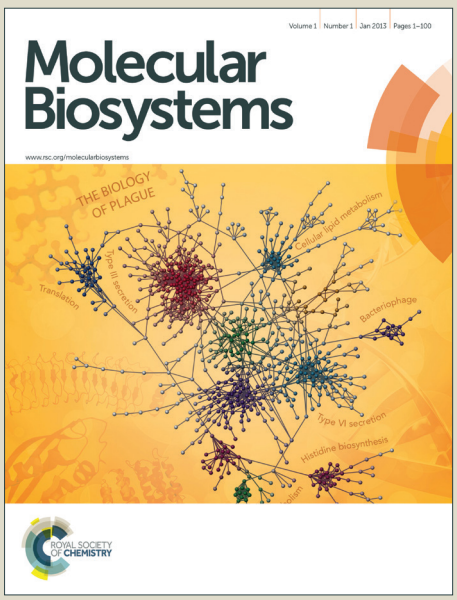

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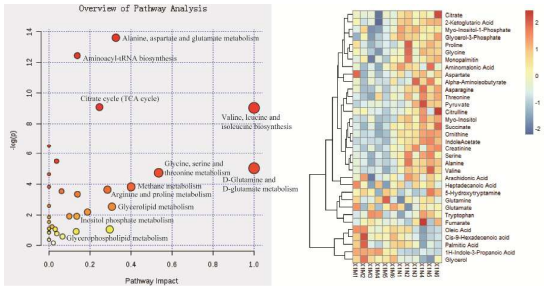
Graphic Abstract



The impact analysis and the heatmap revealed the distinct perturbation effect of methamphetamine on endogenous metabolites and the metabolic pathways.

Metabolic impact of methamphetamine on the systemic metabolism of rats and potential markers of methamphetamine abuse

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ABSTRACT:

Although the stimulating and psychotropic effects of methamphetamine (METH) on the nervous system are well documented, the impact of METH abuse on biological metabolism and the turnover of peripheral transmitters are poorly understood. Metabolomics has the potential to reveal the effect of METH abuse on systemic metabolism and potential markers suggesting the underlying mechanism of toxicity. In this study, Male Sprague Dawley rats were intraperitoneally injected with METH at escalating doses mg/kg for 5 consecutive days and then were withdrawn for 2 days. The metabolites in the serum and urine were profiled and the systemic effects of METH on metabolic pathways were evaluated. Multivariate statistical analysis showed that METH caused distinct deviations, whereas the withdrawal of METH restored the metabolic patterns towards baseline. METH administration elevated energy metabolism, which was manifested by the distinct depletion of branched-chain amino acids, accelerated tricarboxylic-acid cycle and lipid metabolism, reduced serum glycerol-3-phosphate, and elevated serum and urinary 3-hydroxybutyrate and urinary glycerol. In addition to the increased serum levels of the excitatory amino acids glutamate and aspartate (the inhibitory neurotransmitters in the brain), a marked decline in serum alanine and glycine after METH treatment suggested the activation and decreased inhibition of the nervous system and hence elevated nervous activity. Withdrawal of METH for 2 days efficiently restored all but a few metabolites to baseline, including serum creatinine, citrate, 2-ketoglutarate, and urinary lactate. Therefore, these metabolites are potential markers of METH use, and they may be used to facilitate a diagnosis of METH abuse.

Key words: gas chromatography mass spectrometry; methamphetamine; metabolic perturbation; toxicity; metabolomics

Introduction

Methamphetamine (METH) is a well-known drug of abuse that has caused serious problems for society [1, 2]. In recent years, the use of METH has increased markedly all over the world. This epidemic has had substantial effects in regard to public health, psychiatric comorbidity, and economic costs[3]. METH is more potent than its parent compound, amphetamine, because of its lipophilic nature. This structure allows METH to have greater penetration of the central nervous system (CNS) [3]. METH is a highly addictive stimulant that has significant effects on the nervous system [4, 5]. METH induced dose-dependent reductions of DA, 5-HT and TH (tyrosine hydroxylase), and increased GFAP (glial fibrillary acidic protein) [6] and high-dose METH treatment causes damage to dopamine and serotonin terminals in the brains of laboratory animals [7, 8]. Tissue levels of dopamine [9, 10] and serotonin [9] are decreased, while levels of glutamate and dopamine in the nucleus accumbens decrease with the administration of METH [11]. It has been reported that the norepinephrine neurons play an important role in promoting the METH toxicity [12]. Furthermore, the toxicity of METH can lead to autonomic nervous system (ANS) dysfunction, cardiovascular pathology and neurotoxicity [13]. METH can induce dysfunction of the liver, heart and kidney [14]. It was reported that METH abusers suffer cognitive deficits and have the potential to develop metabolic syndrome [15] and hyperthermia during chronic METH use [16], which has strong effects on energy metabolism. The effect of METH on energy metabolism may play an important role in assessing the side effects and toxicity of METH [17]. Unfortunately, although the stimulating and psychotropic effects of METH on neurotransmitters and the nervous system are well known [18], the effects of METH abuse on biological metabolism and the circulatory system in the whole body are poorly understood [19, 20].

Metabolomics is the study of metabolism at the global level. This rapidly developing new discipline has important potential implications for pharmacologic science. It has proven to be a fast and reproducible method that directly reflects biological events [21-23]. Metabolomic studies capture global biochemical events by assaying thousands of small molecules in cells [24-26], tissues [27], organs, or biological fluids, followed by the application of informatics techniques to define metabolomic

signatures. Because metabolites are intrinsically involved in multiple metabolic pathways *in vivo*, the relative quantitation of these metabolites in body fluids can provide a comprehensive profile of the metabolic status of an organism and reveal potential markers of toxicity [28]. Metabolomics can lead to an enhanced understanding of mechanisms for a disease, a drug or a xenobiotic effect, and an increased ability to predict individual variations in drug response phenotypes [22, 29]. Previous studies have shown that metabolomics have been successfully applied to characterizing metabolic features of diseases such as high-altitude pulmonary edema [30], hepatocarcinoma [31, 32], lung cancer [33, 34], colorectal cancer [34], diabetes [35-37], depression [38] and cardiovascular disease [39] among others. In this study, METH-treated rats were used as a model, and the endogenous metabolites in their serum and urine were profiled using a gas chromatography mass spectrometry (GC/MS)-based metabolomics platform. We aimed to evaluate the potential toxicity of METH by characterizing the perturbation effects of METH on metabolic pathways and to identify potential markers of METH abuse.

Results and Discussion

METH can be rapidly distributed in the various tissues and enter the nervous system, and then be quickly eliminated from the body [40]. Animals receiving METH showed elevated locomotor activity compared to baseline over a short period following injection as previously reported [41], and this result was consistent with long-standing observations [42-44]. The physical effects of METH include twitching and muscle weakness, anorexia, behavioral aberrations, hyperactivity, increased movement, numbness, tremors, and rapid breathing [45-49].

GC/MS chromatograms and overview of the serum data

Typical GC/MS chromatograms of the sera from both the METH-treated group and the control group are shown in Figure S1. Visual inspection of the chromatograms revealed differences between the METH-treated rats and the control, e.g., peaks 1 (lactate), 3 (alanine), 5 (urea), 14 (pyroglutamate), and 16 (glutamate). By comparing the mass spectrum of each peak with that available in the libraries and that of the reference compound, a total of 136 compounds were identified, including amino acids, organic acids, amines, saccharides and fatty acids (Table S1). To gain an overview of the data set, an unsupervised PCA model was applied. No outliers were found in the PCA model. A PLS-DA model was then calculated with the samples classified into four groups: the METH-treated rats on day 1 and day 5, after withdrawal of METH for 2 days, and the blank control. The scores plot shows that samples from the same group tended to cluster closely, whereas samples from different groups scattered separately (Figure 1). Treatment with METH for 1 (D1) and 5 days (D5) showed distinct deviation from the control (Figure 1A), suggesting that METH perturbed the sera metabolome of rats. Withdrawal of METH for two days (D5+2) restored the plots of these rats to values close to those of the control, yet not overlapping with the control sera data. These data further indicated that, after the withdrawal of METH for 2 days, the metabolic perturbation of the rats continued to occur, and the rats needed a longer time for the gradual return of the perturbed metabolism towards baseline.

GC/MS chromatograms and overview of the urine data

The typical GC/MS chromatograms for urine showed the metabolite differences between the METH-administered group and the control group (Figure S2). A total of 109 compounds were identified (Table S2): for example, peaks 1 (lactate), 5 (3-hydroxybutyrate), 8 (succinate), 15 (creatinine), 20 (cysteine) and 24 (urate). The PLS-DA model of the urine data showed a pattern similar to that of the serum data (Figure 1B). In general, exposure to METH for 5 days (D5) severely deviated the metabolic events away from the control values, and after the withdrawal of METH for 2 days (D5+2), the scores plot of the METH-treated rats was restored to similarity with the control (Figure 1B). Compared with the scores plot of the sera data (Figure 1A), the scores plot of the PLS-DA model showed that urine samples closely overlapped with those of the control (Figure 1B) after withdrawal of METH for 2 days, suggesting an efficient restoration of urine metabolites to baseline levels. Furthermore, these findings indicated normal kidney function for both the filtration of metabolites from blood and the re-absorption of molecules from blood filtrate in the kidney tubules.

Metabolic effects of METH treatment on sera and urinary metabolites

As shown in figure 1A, the intraperitoneal injection of METH induced program-dependent (treatment and withdrawal) metabolic patterns based on the serum data. After exposure to METH for 1 day, the scores plots obviously deviated from those of the normal control, and exposure of the rats to METH for 5 days caused further deviation, indicating perturbation of the metabolism. Identification of the metabolites revealed that the levels of many amino acids significantly decreased after acute exposure to METH (Figure 2, Table 1): for example, glycine, alanine, ornithine, asparagine, valine, isoleucine, leucine, serine, proline, threonine, methionine and citrulline. Other amino acids, such as tryptophan, glutamine, glutamate, aspartate and lysine, increased with the administration of METH. Continual administration for five days further perturbed the levels of alanine, glycine, lysine, threonine, ornithine, hydroxyproline and citrulline (Table 1), indicating that these metabolites are involved in the acute stress stimuli of METH.

The first intraperitoneal injection of METH decreased the sera levels of intermediates in the tricarboxylic acid (TCA) cycle, such as citrate, 2-ketoglutarate, succinate, fumarate, malate and pyruvate (Figure 2, Table 1). Notably, few of the above metabolites were restored to normal levels after administration of METH for 5 continuous days (Table 1). On the contrary, urinary fumarate, pyruvate, succinate and citrate were obviously elevated on day 5 (Table S3). In serum, lactate decreased, while in urine, its level increased significantly, and the trend continued with the extension of METH administration (Tables 1 and S3).

It is interesting to note that in serum, myo-inositol and myo-inositol-1-phosphate declined gradually (Table 1, Figure 2) to lower than normal levels after the first injection and remained at a low level with the extension of the time of administration. We also found that the urine level of myo-inositol was significantly elevated on day 5. Myo-inositol and myo-inositol-1-phosphate are intermediates involved in inositol phosphate metabolism and the phosphatidylinositol signaling system [50, 51], and their trends were opposite to those for heroin [52].

With regard to the metabolites in tryptophan metabolism, the sera level of indoleacetate increased, while those of tryptophan and 5-hydroxytryptamine were not significantly perturbed (Figure 3, Table 1). After the administration of METH for 5 days, the urine level of 5-hydroxyindoleacetic acid was obviously decreased (Table S3), and its trend was different from that invoked by heroin [52].

Treatment with METH also affected the turnover of lipids and free fatty acids (FFAs) (Table 1, Figure 4). Although the sera levels of monopalmitin and glycerol-3-phosphate decreased quickly after the first administration of METH, the serum levels of most FFAs, such as arachidonic acid, palmitic acid, oleic acid, cis-9-hexadecenic acid, decanedioic acid, stearic acid, and heptadecanoic acid, were not significantly decreased until continuous treatment with METH for five days. In urine, the levels of palmitic acid and stearic acid decreased on day 5 and maintained the same trend with the withdrawal of METH for 2 days. Both sera and urinary 3-hydroxybutyrate were obviously elevated after the administration of METH, and the levels of some FFAs, such as palmitic acid and stearic acid,

decreased slowly with the administration of METH. These results indicated that the degradation of fatty acids accelerated and the consumption of lipids increased. It was further suggested that the turnover of free fatty acids play a key role in depicting the evolution progress of energy metabolism, i.e., starting with the enhanced catabolism of glycerol-lipid (e.g., monopalmitin and glycerol-3-phosphate) and generation of FFA, and subsequently, the increased β-oxidation of FFA and the formation of the 3-hydroxybutyrate.

Restoration of perturbed metabolism after withdrawal of METH

For better understanding the metabolic perturbation induced by METH, a withdrawal program was designed to (1) identify the metabolites that did or did not restore to baseline, (2) study the potential association between the perturbed endogenous metabolites/metabolic pathway and the METH induced toxicity. Generally, the withdrawal of METH significantly restored the metabolic pattern of the METH-administered rats towards that of the non-treated controls, according to the PLS-DA model (Figure 1A, B). A direct comparison of the sera data showed that the withdrawal of METH for 2 days reversed the metabolic perturbation, and most of the serum metabolites, such as asparagine, aspartate, glutamate, glutamine, citrulline, proline and threonine, were restored to baseline after the two-day withdrawal (Table 1), and serum levels of arachidonic acid, decanedioic acid, stearic acid and glycerol-3-phosphate recovered to some extent. However, in serum, levels of isoleucine, palmitic acid, creatinine, citrate and 2-ketoglutarate were not effectively restored. Similarly, in urine, most perturbed metabolites, such as serine, glutamate, alanine, 3-hydroxybutyrate, glycine, glycerol, pyruvate, succinate, citrate, fumarate, myo-inositol and 5-hydroxyindoleacetic acid, were restored to baseline after the withdrawal of METH for 2 days (Table 2), yet urinary lactate was maintained at a significantly higher level than in the controls (Figure S3, Table S3).

Difference of the metabolic perturbation induced by METH and heroin

GC/MS analysis of metabolites in serum and urine can profile many molecules involved in energy metabolism, such as branched-chain amino acids, fatty acids, carbohydrates, intermediates in the TCA

cycle, and neurotransmitters such as 5-hydroxytryptamine, glutamate, and others [53, 54]. Although, regretfully, this study did not employ the developed method to profile more neurotransmitters [55], based on the established metabolomic platform and GC/MS techniques [56], most of the above molecules and totals of 136 and 109 metabolites were detected in serum and urine samples, respectively. In this study, multivariate statistical analysis showed that METH induced distinct metabolic perturbation in the rats, although the perturbation was obscured after withdrawal of METH for two days. Generally, administration of METH affected metabolic patterns based on both sera and urinary data, but withdrawal of METH efficiently restored urinary metabolites back to baseline levels, while metabolites in serum were still deviated, according to the metabolic pattern of the sera data (Figure 1).

Compared with the research we have conducted on heroin, the metabolic changes induced by METH abuse were minor [52], and they recovered more quickly. This result indicates that the effects of heroin on the body are greater than those of METH. The change trends of many compounds varied after administration of the two drugs, e.g., those of most amino acids and some intermediate metabolites in serotonin metabolism [52]. The declining trends for the serum levels of leucine, valine, and threonine and the trends of elevation for tryptophan induced by METH (Table 1, Figure 2) were opposite to those invoked by heroin. The influence of METH on the TCA intermediates was greater than that of heroin, and their trends were in opposition [52]. METH showed the reverse effect on inositol phosphate metabolism from that of heroin [52]. However, after METH administration, FFA and lipid metabolism followed the same trend as for heroin, with reduced serum levels of FFAs and lipids, such as palmitic acid and oleic acid [52]. The discrepancies between the effects of METH and heroin on these metabolites might be attributable to variations in the psychotropic activity of METH and heroin on metabolism and in the sampling time points.

Effects of METH on energy metabolism

Based on the discriminant metabolites showed in the heatmap, the metabolic pathway impact analysis

and the enrichment overview revealed the distinct perturbation of METH to amino acid metabolism, the citrate cycle, inositol phosphate metabolism and glycerolipid metabolism [57, 58] (Figure 5). The data clearly showed the effects of METH on metabolic pathways and that the withdrawal of METH restored most of the metabolites to baseline levels (Figure 5 G, I). It was interesting to note that after the first dose of METH, distinct changes of metabolites in serum were observed (Figure 5 A~C). With the continued administration of METH, the difference between the treated rats and the controls became smaller (Figure 5 D~F, H). In general, administration of METH greatly perturbed energy metabolism. As the first evidence, levels of branched-chain amino acids, i.e., valine, leucine and isoleucine, sharply declined with exposure to METH, suggesting their consumption in large quantities for energy supply. It was reported that valine deficiency is marked by neurological defects in the brain, while isoleucine deficiency is marked by muscle tremors [59]. Their levels clearly explained the fact that the rats' behavior was more active, and that they exhibited muscle tremors, which is consistent with increased physical activity in rats. Moreover, the continued administration of METH for 5 days also resulted in lower levels of metabolites of glycolysis and the TCA cycle (glucose, pyruvate, citrate, and 2-ketoglutarate; Table 1), suggesting the down-regulation of glycolysis and the TCA cycle and the rapid depletion of carbohydrates after intense activity by the rats [60, 61]. As an important energy source, lipid metabolism was also gradually perturbed. On the first day, injection of METH reduced glycerol phosphate and monopalmitin, typical lipids for source energy, yet significantly elevated sera glycerol, suggesting the increased degradation of glycerol lipids. Meanwhile, the levels of most fatty acids were not significantly perturbed on the first day. These results suggested a balance in the turnover of fatty acids between the decomposition of lipid esters and the metabolism of fatty acids. Longer and continuous treatment with METH reduced serum levels of free fatty acids, such as arachidonic acid, oleic acid, palmitic acid, heptadecanoic acid, and cis-9-hexadecenoic acid; while in urine, levels of 3-hydroxybutyrate and glycerol increased, suggesting the elevated β-oxidation of fatty acids and the decomposition of glycerol phosphate (Figure 4).

Effects of METH on neurotransmitters

Apart from energy metabolism, metabolites involved in neurotransmission were perturbed. As the excitatory amino acids, higher levels than usual of aspartate and glutamate in the central nervous system suggest excitation of the animals, while lower levels indicate inhibition of the central nervous system. Previous studies showed that METH caused a significant decrease in the glutamate content of the striatum, hippocampus and midbrain, an increase in the aspartate content of the hypothalamus [62], and that the combined concentrations of glutamate and glutamine in the frontal white matter increased [63, 64], while the ambulation-increasing effect of METH was augmented by pretreatment with glutamate and aspartate at 30 min before METH administration [65]. Generally, although the metabolites in the nervous system are not available, increased levels of aspartate and glutamate in the serum suggest an increase of nervous activity, which is consistent with the increased activity of rats after administration of METH. On the other hand, like GABA, taurine and glycine, alanine is the other inhibitory neurotransmitter in the brain [66]. The markedly decreased sera levels of alanine and glycine suggested the reduced inhibition of the nervous system and the elevated nervous activity. Furthermore, as the inhibitory metabolite for nervous activity, 5-hydroxytryptamine in serum was not significantly perturbed (Figure 3, Table 1). However, it was determined that its major metabolites, 5-hydroxyindoleacetic acid in urine and indoleacetate in serum, all decreased rapidly after METH administration. The association of these metabolites to the peripheral circulation and the central nervous system and the effects of the two metabolites on the nervous system remain to be clarified.

Myo-inositol-1-phosphate is a metabolite involved in inositol phosphate metabolism and in the phosphatidylinositol signaling system. It is a breakdown product of phosphatidylinositol, and examining changes in the levels of myo-inositol-1-phosphate may provide a means of noninvasively monitoring phosphatidylinositol metabolism in vivo [67]. It was reported that METH-exposed children had lower levels of myo-inositol in the thalamus, and the reduced myo-inositol suggested lower glial content in the thalamus [63]. In METH abusers, the myo-inositol concentrations were higher in the frontal white matter [68] and frontal grey matter [69] than those in healthy comparison subjects. The sharp decrease in the serum levels of myo-inositol-1-phosphate and myo-inositol and the obviously elevated level of myo-inositol in urine (Figure 2 and 3, Table 1) suggested the disturbance by METH of

phosphatidylinositol and inositol phosphate metabolism and indicated the aberrant neuronal and glial development in these brain regions [63].

Conclusions

In this study, we demonstrated the disturbance of METH to the metabolism. Treatment with METH caused distinct deviations from the control, whereas the withdrawal of METH restored the metabolic patterns towards baseline. METH administration elevated energy metabolism, accelerated tricarboxylic-acid cycle and lipid metabolism. And withdrawal of METH for 2 days efficiently restored all but a few metabolites to baseline, including serum creatinine, citrate, 2-ketoglutarate, and urinary lactate. Therefore, these metabolites are potential markers of METH use, even when METH has been withdrawn for several days, and they may be used to facilitate a diagnosis of METH abuse.

Experimental

Materials and reagents

The reference standard METH was purchased from the Jiangsu Institute for Food and Drug Control (Jiangsu, CHINA). The stable-isotope-labeled internal standard compound (IS) myristic-1,2-$^{13}C_2$ acid (99 atom%^{13}C), methoxyamine hydrochloride (purity 98%), and pyridine (≥ 99.8% GC), were provided by Sigma-Aldrich (St. Louis, USA). N-methyl-N-trimethylsilyltrifluoroacetamide and 1% trimethylchlorosilane were purchased from Thermo Scientific (Bellefonte, USA). High-performance liquid chromatography-grade methanol and n-heptane were obtained from Tedia Company (Fairfield, USA) and Merck (Darmstadt, Germany), respectively. Purified water was produced with a Milli-Q system (Millipore, Bedford, USA).

Apparatus

Chromatographic separation of the analytes was achieved with a Shimadzu GCMS-QP2010 (Shimadzu Corp., Tokyo, Japan) equipped with an RTx-5MS column (30 m × 0.25 mm i.d.

fused-silica capillary column chemically bonded with a 0.25 μm crossbond, 5% diphenyl/95% dimethyl polysiloxane, Restek Corporation, PA, USA). A Sorvall Biofuge Stratos centrifuge (Sollentum, Germany) and an SPD2010-230 SpeedVac Concentrator (Thermo Savant, Holbrook, USA) were used to centrifuge the samples and evaporate the supernatant to dryness, respectively.

Animals and samples

The experiments were performed on adult male Sprague Dawley rats weighing 180 ± 20 g (Sino-British Sippr/Bk Lab. Animal Co., Ltd, Shanghai). The rats were housed individually in metabolic cages, with free access to a standard chow diet (China Experimental Animal Food Standard, GB 14924.2–2001 and GB 14924.3–2001) and water under controlled environmental conditions (temperature, 22 ± 2 ℃; humidity, 55 ± 10%). All experimental procedures involving the use of animals complied with the Guidelines for Animal Experimentation of the China Pharmaceutical University (Nanjing, China), and the protocol was approved by the Animal Ethics Committee of that institution.

After adaptation to standard laboratory conditions for a week, the rats were randomly allocated to two groups, a METH-treated group and an untreated control. The METH-treated group was administered intraperitoneal METH in 0.9% saline, and the control was given an equal volume of vehicle. After overnight fastening, an increasing amount of METH was given to the rats for 5 consecutive days at 10, 12.5, 15, 20 and 30 mg/kg body weight [6, 70-72], followed by withdrawal of METH for 2 days. Blood samples were collected from the orbital venous plexus 1 h after administration of METH on days 1 and 5 and after withdrawal of METH for 2 days, and urine was collected 12 h before the blood samples were collected. The serum and urine were prepared and stored at –70 ℃. All serum and urine samples were thawed by incubation at 37 ℃ for 20 min and thoroughly vortexed before extraction.

Sample preparation, derivatization, and GC /MS analysis

The serum and urine samples were pretreated, extracted, and derivatized as reported [53, 73]. Briefly, 50 μL serum was added to 200 μL of methanol containing the internal standard myristic-1,2-$^{13}C_2$ (5 μg/mL) and vigorously vortex extracted. For urine samples, 30 μL urine was first added to urinase to decompose the excessive urea in the urine, then the mixture was added to methanol and vigorously vortex extracted. The supernatants of the sera (100 μL) and urine (60 μL) were then dried, methoxylated, trimethylsilylated, and analyzed with gas chromatography/mass spectrometry (GC/MS). Derivatized samples for GC/MS were separated on a DB-5 column with helium as the carrier gas and a temperature ramp from 80 ℃ to 300 ℃ and then analyzed with electron impact ionization and a 50-700 atomic mass unit scan range [54]. To minimize systematic variations, all samples were analyzed in random order, and the quantitative data were normalized to the internal standard. After GC/MS analysis, compounds were identified by automated comparison of the ion features including retention time, molecular weight (m/z), preferred adducts, in-source fragments, and associated MS spectra of the experimental samples with those of reference standards or those available in libraries, such as mainlib and publib in the National Institute of Standards and Technology (NIST) library 2.0 (2008); Wiley 9 (Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany); the in-house mass spectra library database established by Umeå Plant Science Center (Umeå University, Sweden); and the Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University (Nanjing, China) [74]. Compound abundance was quantified by calculating the area under the curve for the quantification ion of the compound.

Multivariate statistical analysis

The relative quantitative data for the peaks (peak areas) were first normalized against the IS and then subjected to multivariate statistical analysis using SIMCA-P 13 software (Umetrics, Umeå, Sweden) [75]. To minimize the effect of the amount of urine from each rat, the urinary data were normalized to urine volume [76]. The parameters for a principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were specified, and the results were interpreted as described previously [73, 77]. Cross-validation, with seven cross-validation groups and 100 iterations, was used

throughout to determine the number of principal components (PCs). The statistical analysis was performed using one-way analysis of variance (ANOVA) with a significance level of 0.01 or 0.05.

Acknowledgments

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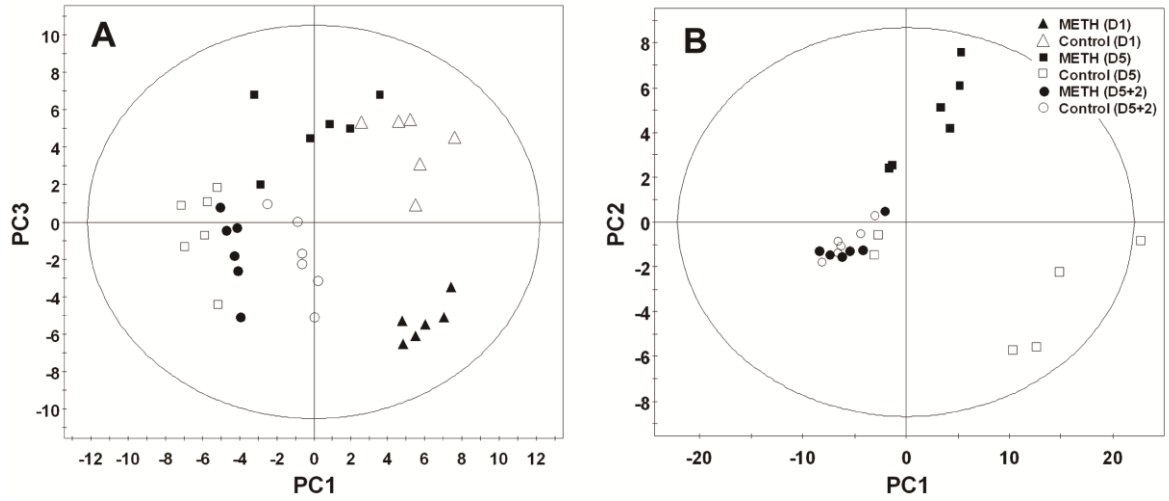

Figure 1 PLS-DA model of metabolic patterns for rats treated with METH. A. Serum data. The five-component PLS-DA model explained 80.9% and predicted 56.9% of the sample variation in Y (sample types) and explained 49.1% of the variation in X (R^2X=0.559, R^2Y=0.809, Q^2Y=0.491, respectively) according to cross-validation. The first and the third components explained and predicted the largest variation in Y (PC1: R^2X=0.167, R^2Y=0.183, Q^2Y=0.145; PC2: R^2X=0.129, R^2Y=-0.021, Q^2Y=0.118; PC3: R^2X=0.246, R^2Y=0.270, Q^2Y=0.142; PC4: R^2X=0.310 R^2Y=0.396, Q^2Y=0.210; PC5: R^2X=0.355, R^2Y=0.0626, Q^2Y=0.268). B. Urine data. The three-component PLS-DA model explained 68.9% and predicted 41.2% of the sample variation in Y (sample types) and explained 83.4% of the variation in X (R^2X = 0.834, R^2Y = 0.689, Q^2Y = 0.412) according to cross-validation. The third and second components explained and predicted the largest sample variation in Y (PC1: R^2X=0.640, R^2Y=0.202, Q^2Y=0.166; PC2: R^2X=0.103, R^2Y =0.259, Q^2Y =0.245; PC3: R^2X=0.150, R^2Y =0.387, Q^2Y =0.189). D1, day 1; D5, day 5; D5+2, withdrawal of METH for 2 days.

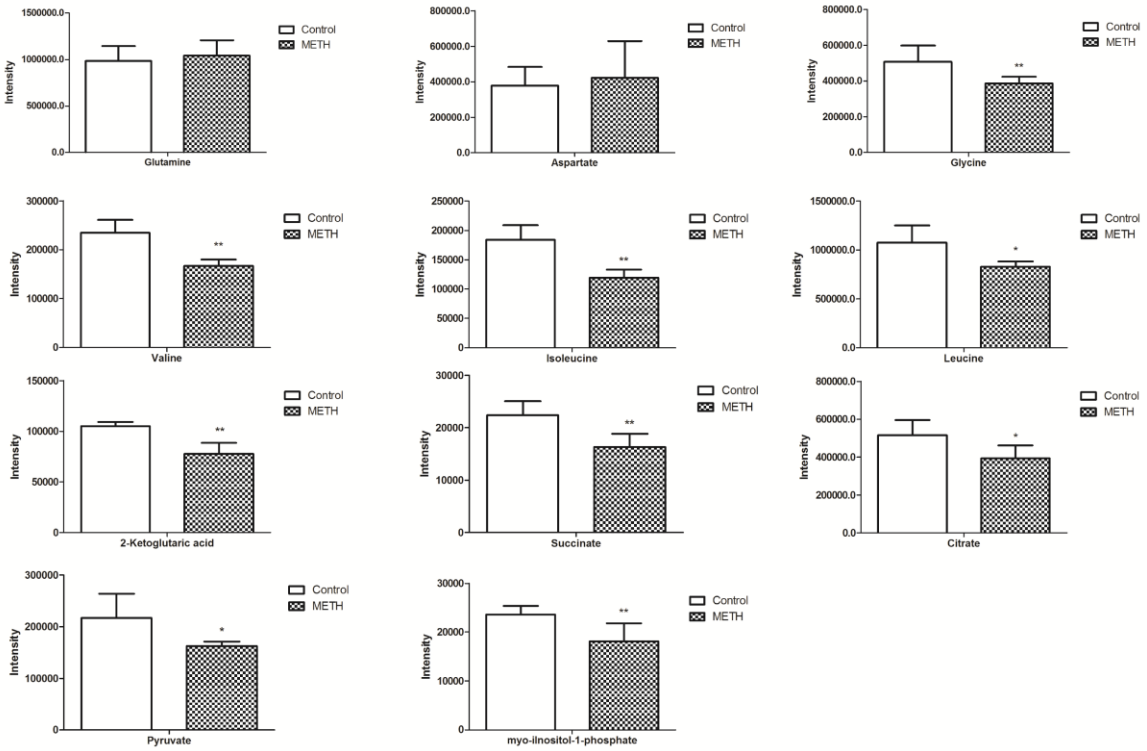

Figure 2 The effects of METH exposure for 3 hours (D1) on metabolites in serum. (*P < 0.05, **P < 0.01, compared with healthy controls)

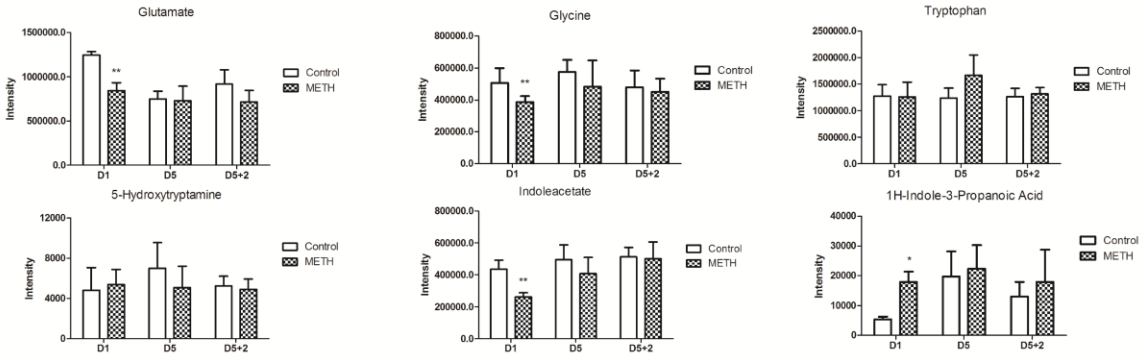

Figure 3 Relative abundance of serum metabolites at 3 different stages. Exposure to METH for 3 h (D1), exposure to METH for 5 days (D5), withdrawal of METH for 2 days (D5+2).(*P < 0.05, **P < 0.01, compared with healthy controls)

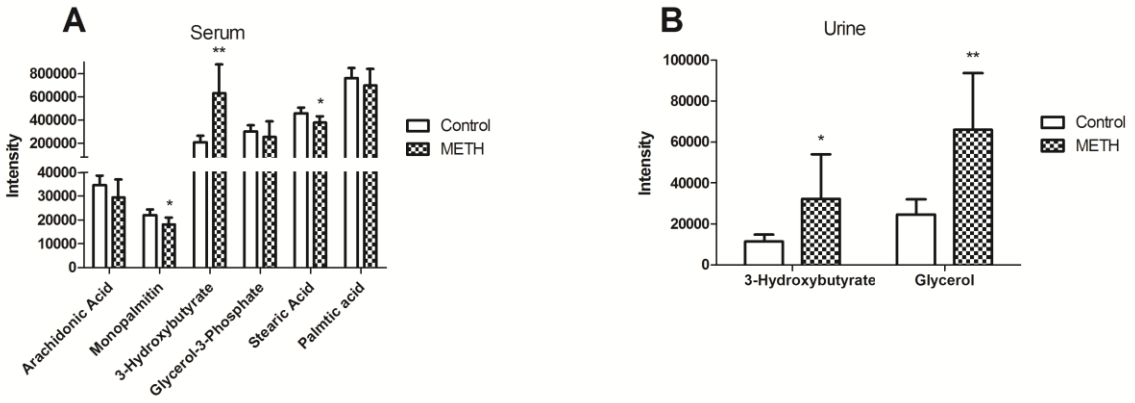

Figure 4 Metabolites of fatty acid metabolism change with exposure to METH for 5 days. A, metabolites in serum; B, metabolites in urine. (*P < 0.05, **P < 0.01, compared with healthy controls)

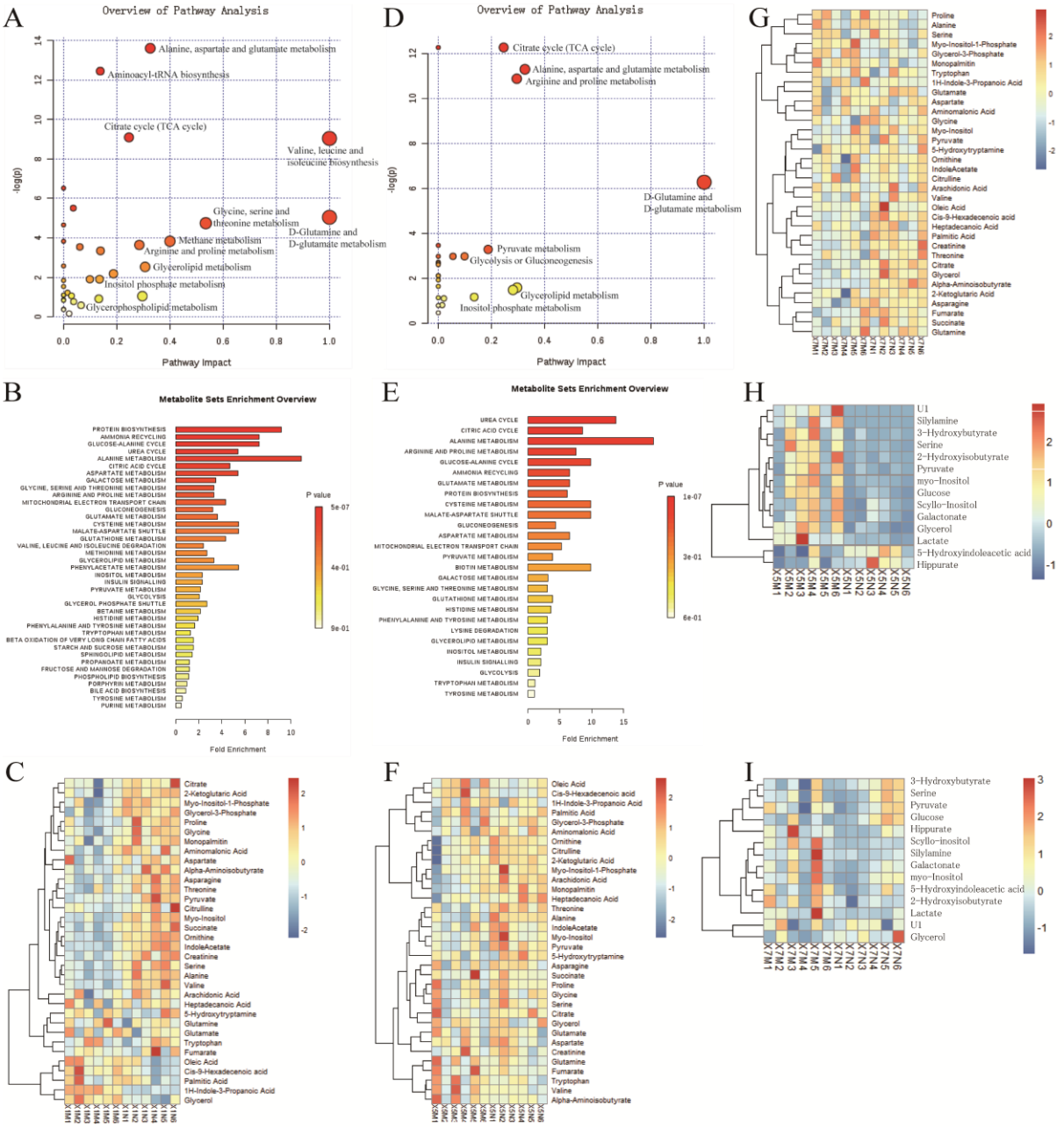

Figure 5 Overview of the impact of METH on metabolites of rats.

A. The pathway impact of METH on sera metabolites, day 1.

B. The enrichment overview of the pathway-associated metabolite sets in serum perturbed by METH, day 1.

C. Heatmap of the serum metabolites perturbed by METH, day 1.

D. The pathway impact of METH on sera metabolites, day 5.

E. The enrichment overview of the pathway-associated metabolite sets in serum perturbed by METH, day 5.

F. Heatmap of the serum metabolites perturbed by METH, day 5.

G. Heatmap of the serum metabolites after withdrawal of METH for 2 days (Day 7).

H. Heatmap of the urinary metabolites perturbed by METH, day 5.

I. Heatmap of the urinary metabolites after withdrawal of METH for 2 days (Day 7).

(1M, METH group, administration for 1 day; 1N, normal group, day 1; 5M, METH group, administration for 5 days; 5N, normal group, day 5; 7M, METH group, withdrawal for 2 days after administration for 5 days; 7N, normal group, day 7)

Table 1

Relative abundance of serum metabolites in rats exposed to METH (n = 6)

Metabolic Pathways	Metabolites	Pre	D1	D5	D5+2
Amino acids	Alanine	248.62 ±32.57	147.11 ±20.15$^{*\#}$	169.25 ±48.49$^{*\#}$	269.36 ±40.73
	Asparagine	6.08 ±0.67	4.57 ±0.42$^{*\#}$	4.69 ±1.78	4.93 ±0.89
	Aspartate	37.67 ±10.70	38.08 ±18.77	45.74 ±13.63	49.73 ±20.10
	Citrulline	1.35 ±0.30	1.10 ±0.10$^{*\#}$	0.98 ±0.33$^{*\#}$	1.08 ±0.29
	Glutamate	59.39 ±16.78	90.02 ±10.76$^{\#}$	77.86 ±14.21	75.58 ±8.81
	Glycine	54.84 ±5.94	38.61 ±3.75$^{*\#}$	45.22 ±3.28*	45.07 ±8.28*
	Glutamine	96.80 ±13.47	104.20 ±16.49	119.38 ±35.50	84.51 ±17.21
	Proline	127.04 ±15.39	99.80 ±12.01$^{*\#}$	98.03 ±19.94$^{\#}$	121.68 ±19.13
	Ornithine	19.21 ±1.35	14.06 ±1.18$^{*\#}$	13.98 ±5.19$^{*\#}$	14.15 ±4.89
	Serine	104.65 ±15.55	74.38 ±8.62$^{*\#}$	78.95 ±22.25	87.21 ±8.92
	Threonine	28.15 ±3.21	23.06 ±3.20$^{*\#}$	21.81 ±6.22$^{*\#}$	26.13 ±3.17
	Tryptophan	119.67 ±27.10	121.26 ±27.65	146.74 ±46.54	132.02 ±11.74
	Valine	23.48 ±2.66	16.68 ±1.35$^{*\#}$	18.81 ±6.15	15.54 ±2.62
	Leucine	107.50 ±17.62	82.80 ±5.55$^{*\#}$	88.38 ±26.39	73.98 ±13.10
	Isoleucine	18.39 ±2.50	11.93 ±1.39$^{*\#}$	12.95 ±2.86$^{\#}$	12.28 ±1.75$^{*\#}$
	Hydroxyproline	43.06 ±5.95	38.65 ±3.89$^{\#}$	27.82 ±9.72$^{*\#}$	32.52 ±6.14
	Taurine	6.07 ±2.72	3.91 ±2.39$^{\#}$	4.58 ±2.61	4.25 ±1.68
	Methionine	1.97 ±0.16	1.65 ±0.13$^{*\#}$	1.59 ±0.38$^{\#}$	1.79 ±0.28
	Lysine	235.29 ±38.18	295.04 ±59.24$^{\#}$	297.78 ±75.01$^{*\#}$	342.49 ±4.18$^{\#}$
	Ketoleucine	18.39 ±2.50	11.93 ±1.39$^{*\#}$	12.95 ±2.86	12.28 ±1.75
FFAs and lipids	Monopalmitin	2.39 ±0.34	1.82 ±0.38$^{*\#}$	1.81 ±0.29$^{*\#}$	2.28 ±0.35*
	Monostearin	2.00 ±0.22	1.75 ±0.37	1.62 ±0.34	2.01 ±0.26
	Arachidonic acid	3.96 ±0.59	3.49 ±0.79	2.95 ±0.75	3.54 ±0.53
	Oleic acid	22.60 ±5.14	29.08 ±4.94	19.80 ±7.57	17.40 ±4.01
	Palmitic acid	100.52 ±13.77	109.09 ±16.93	69.70 ±14.38$^{*\#}$	68.47 ±10.35$^{*\#}$
	Heptadecanic acid	1.11 ±0.14	1.05 ±0.24	0.73 ±0.08$^{\#}$	0.82 ±0.11
	Cis-9-Hexadecenoic acid	4.41 ±1.10	6.87 ±1.61$^{*\#}$	3.17 ±1.38$^{\#}$	1.42 ±0.59$^{*\#}$
	3-Hydroxybutyrate	20.61 ±5.73	115.78 ±12.62$^{\#}$	63.17 ±24.70$^{*\#}$	11.82 ±1.49
	Decanedioic acid	3.54 ±0.50	3.16 ±0.47	3.24 ±0.59	3.37 ±0.33
	Stearic acid	53.84 ±4.32	47.93 ±6.47	38.00 ±5.40$^{*\#}$	43.36 ±5.40
	Glycerol	15.16 ±2.05	19.82 ±1.85$^{\#}$	13.99 ±2.41	11.36 ±1.57
	Glycerol-3-Phosphate	38.99 ±3.23	28.55 ±5.63$^{\#}$	25.24 ±13.61$^{\#}$	34.91 ±6.80
Organic acid	Alpha-Aminoisobutyrate	15.10 ±3.65	11.82 ±1.81	5.53 ±2.37$^{\#}$	3.01 ±0.34$^{*\#}$
	Aminomalonic acid	3.23 ±0.82	2.26 ±0.63$^{*\#}$	3.14 ±1.15	3.37 ±0.53

TCA intermediates	2-Ketoglutarate	10.48±0.44	7.78±1.07$^{\#}$	6.62±1.90$^{\#}$	6.32±1.07$^{*\#}$
	Citrate	51.55±8.07	39.31±6.94$^{*\#}$	28.34±8.18$^{\#}$	17.61±5.12$^{*\#}$
	Fumarate	1.69±0.73	1.49±0.21	2.12±1.04	1.42±0.47
	Malate	2.60±0.57	2.08±0.18	2.45±1.49	1.45±0.62
	Pyruvate	21.64±4.70	16.21±0.88$^{*\#}$	14.07±4.51$^{*\#}$	17.41±1.99
	Succinate	2.23±0.27	1.63±0.25$^{*\#}$	2.08±1.70	1.18±0.34$^{\#}$
Others	Galactonolactone	2.33±0.71	4.20±1.28$^{*\#}$	5.29±2.55$^{\#}$	8.50±1.31$^{*\#}$
	Gluconic acid	1.29±0.31	1.56±0.61	2.07±1.07	1.61±0.24
	Glucose	1803.15±285.23	1468.80±370.30	1480.72±538.53	1940.11±312.26
	Creatinine	15.50±3.44	10.17±1.74$^{\#}$	9.40±4.72$^{\#}$	6.97±2.89$^{*\#}$
	Indoleacetate	43.52±5.55	26.11±2.65$^{\#}$	40.77±10.07	49.95±10.56
	5-Hydroxytryptamine	0.48±0.22	0.51±0.15	0.51±0.21	0.49±0.10
	Myo-Inositol	28.28±2.51	20.03±1.81$^{\#}$	21.64±3.35	23.13±4.09
	Myo-Inositol-1-Phosphate	2.36±0.18	1.81±0.37$^{\#}$	1.75±0.59$^{\#}$	2.47±0.64
	Lactate	281.60±54.74	185.89±24.29$^{\#}$	237.16±79.56	229.63±55.88

Note: Peak areas of the metabolites were normalized against those of the internal standard, and the data are given as the mean ± SD. Pre, pre-administration of METH; D1, exposure to METH for 3 h; D5, exposure to METH for 5 days; D5+2, withdrawal of METH for 2 days.

* $P < 0.05$, significantly different compared with control;

$^{\#}$ $P < 0.05$, significantly different compared with Pre.