Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 30 No. 3, 122-129, 2017
https://doi.org/10.5806/AST.2017.30.3.122

Comparative analysis of urinary metabolites in methamphetamine self-administrated rats

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(Received March 7, 2017; Revised April 25, 2017; Accepted May 4, 2017)

Abstract: Methamphetamine addiction is a critical issue due to the lack of effective pharmacotherapy and high potential for relapse. Nevertheless, there are no distinct biomarkers for diagnosis or prognosis for methamphetamine addiction. In the present study, a rat model for methamphetamine self-administration was established and alteration of urinary metabolites by methamphetamine addiction was investigated by the targeted metabolite analysis using mass spectrometry. Rat urine samples were collected at three time points (before and after addiction and after extinction) from the methamphetamine-addicted group as well as the age-matched control group. The collected samples were prepared using Absolute*IDQ* p180 kit and analyzed using flow injection analysis (FIA) - or high performance liquid chromatography (HPLC) - tandem mass spectrometry (MS/MS). The levels of lysine, acetylornithine and methioninesulfoxide were distinctively altered depending on the status of metheamphetamine addiction or extinction. In particular, the level of acetylornithine was reversely changed from addiction to extinction, for which further studies could be useful for biomarker discovery or mechanistic studies for methamphetamine addiction.

Key words: methamphetamine, self-administration, metabolite, mass spectrometry, acetylornithine

1. Introduction

Methamphetamine is a psychostimulant that has serious abuse potential and neurotoxic effects, mainly caused by the release of central and peripheral monoamines.[1] Methamphetamine abuse has been a serious social problem because of its increasing use since the 1990s. It is reported that methamphetamine is the second most abused substance following cannabis and is calculated that 0.3-1.3 % of the world population abuse methamphetamine.[2,3] Clinically, methamphetamine addiction is a critical issue due to lack of effective pharmacotherapy and a high potential for recurrence.[4] Furthermore, no distinct biomarkers for diagnosis or prognosis for drug addiction have yet been found.

Metabolomics, an endpoint of the omics cascade, is a powerful tool in understanding the comprehensive

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changes in metabolic responses in living systems induced by external stimuli or genetic alteration. Unlike genomics, transcriptomics and proteomics, metabolomics reflects the phenotype of living things, and enables to measure the state of many metabolites and discover related biomarkers.[5,6] Mass spectrometry (MS) is a powerful tool with high sensitivity and reproducibility in metabolomics.[7] In particular, the targeted metabolite analysis using MS provides reliable outcome for quantitative changes for selected metabolites.

Self-administration is a superior animal model for drug abuse as it is very reliable and has compelling validity.[8] The self-administration process offers a direct relation to addictive behavior, happening in the natural environment with features of human drug abuse modeled by the self-administration process in animals.[9] For this reason, this model can offer an understanding of factors that are affected during addiction, and provides a potential for therapeutic treatments.[8,9] There have been several published reports of cocaine, morphine, and nicotine addiction using self-administration.[10-12] However, little have been reported about metabolic changes after self-administration of addictive drugs, including methamphetamine.

In the present study, a rat model for methamphetamine self-administration was established to investigate alteration of urinary metabolites by methamphetamine addiction. The targeted urinary metabolites between before and after methamphetamine addiction were quantitatively compared with their age-matched groups using flow injection analysis (FIA) - or high performance liquid chromatography (HPLC) - tandem mass spectrometry (MS/MS).

2. Materials and Methods

2.1. Materials

Methamphetamin hydrochloride was obtained from the Ministry of Food and Drug Safety (Chungcheongbuk-do, Republic of Korea). Absolute*IDQ* p180 kit for the HPLC-MS analysis was purchased from Biocrates Life Sciences AG (Innsbruck, Austria). All solvents were of HPLC grade and other chemicals were all a reagent grade.

2.2. Animals

Fourteen male Sprague-Dawley (SD) rats (11 weeks of age) weighing 310-350 g were purchased from Daehan Animal (Seoul, Republic of Korea) and were kept in the laboratory animal facility with a 12 h light/dark cycle. All rats were provided with tap water and a commercial diet ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at Daegu Haany University.

2.3. Methamphetamine self-administration and urine sample collection

Methamphetamine self-administration was conducted as previously described[12,13] with some modification. Briefly, rats were surgically implanted with chronic indwelling jugular catheters (Dow Corning, Midland, MI, USA; inner diameter, 0.02 in.; outer diameter, 0.037 in.) into the right jugular vein and were allowed to recover for six days. Then, the rats were trained to press a lever for 45 mg food pellets (Bio-Serv, Frenchtown, NJ, USA) in the operant chambers housed in sound attenuated cubicles (Med Associates, St. Albans, VT, USA) until the food criteria had been achieved (100 food pellets for three consecutive days) in 3 h daily sessions.

After food training, the rats were divided into two groups, an age-matched control group (C, n = 7) and methamphetamine-addicted group (M, n = 7). Then, each rat was separately housed in a metabolic cage and urine was collected into an ice-bathed container for 12 hours. Those urine samples were designated as C1 or M1. Then, the chamber was prepared to infuse intravenously saline (for C) or methamphetamine solution dissolved in saline (0.05 mg/kg/injection for M) into the intravenous catheter of the rat when pressing the lever for self-administration. For the rat of the M group, the methamphetamine self-administration was performed in daily 2-h sessions until the rats showed stable responding with the average number of the lever press in three consecutive sessions with less than 20 % variation. For the rat of the C

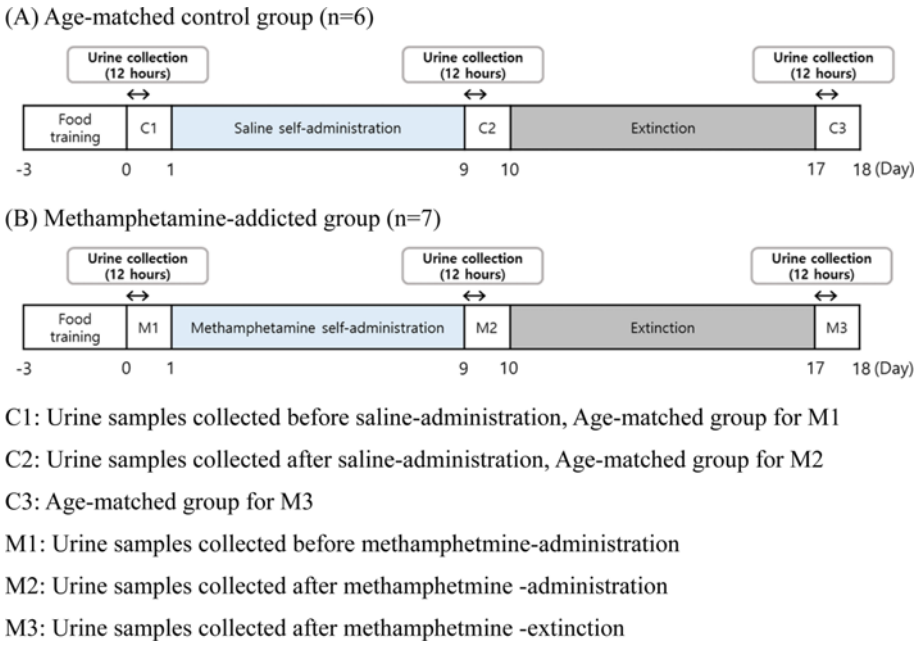

Fig. 1. Time points for urine collection from rats during saline- or methamphetamine self-administration and group names of urine samples.

group, the saline self-administration was performed for the same period as the M group. At the end of the self-administration, urine was collected into an ice-bathed container in a metabolic cage for 12 hours (C2 or M2). After the collection of the urine samples, the rats of the M group received saline in the chamber until the rats showed stable extinction responding. For the rat of the C group, the saline self-administration was kept going. Next day, urine was collected into an ice-bathed container in a metabolic cage for 12 hours (C3 or M3). The time points for urine collection from rats during saline- or methamphetamine self-administration and the group names of urine samples are displayed in *Fig.* 1.

2.4. Urine sample preparation

Absolute*IDQ* p180 kit, which allow the simultaneous quantification of a total of 186 metabolites (40 acylcarnitines, 41 amino acids and biogenic amines, 90 glycerophospholipids, 15 sphingolipids) and a sum of hexoses including glucose, was used for urine sample preparation.[14] Ten microliters of each urine sample with 10 μL of the mixture solution of internal standards, composed of 46 isotope-labeled and chemically homologous internal standards, was loaded into the kit, derivatized and extracted using a protocol provided by the manufacturer.[14] After filtering on the kit, the filtrate was injected onto both FIA- and HPLC-MS/MS.

2.5. Instrumental analysis

An AB Sciex 4000 QTrap mass spectrometer (Sciex, Framingham, MA, USA) in the multiple reaction monitoring detection mode with electrospray ionization was used for analysis at Inha University Hospital Clinical Trial Center (Incheon, Korea). Forty microliters of the prepared samples were injected to MS/MS by FIA for the determination of the group of amino acids and biogenic amines and 10 μL of those samples were introduced by HPLC to MS/MS for the determination of the other groups. The kit was validated using MetValTM (Biocrates Life Scicences AG) software and the analytical results were processed using AnalystTM (Sciex) and MetValTM software.

For the HPLC-MS/MS analysis, the Zorbax Eclipse XDB C18 column (3.0 mm × 100 mm, 3.5 μm; Agilent)

was used. The mobile phase comprised 0.2 % formic acid in water (A) and 0.2 % formic acid in acetonitrile (B) and had the following gradient condition: 0-0.5 min, 0 % (B); 0.5-5.5 min, 0-95 % (B); 5.5-6.5 min, 95 % (B); 6.5-7 min, 95-0 % (B); and 7-9.5 min, 0 % (B). The flow rate was 0.5 mL/min. For the FIA-MS/MS analysis, the sample was run with the solvent provided by the manufacturer at following rates: 0-1.6 min, 0.03 mL/min; 1.6-2.4 min, 0.2 mL/min; 2.4-2.8 min, 0.2 mL/min; 2.8-3.0 min, 0.03 mL/min.

2.6. Statistical analysis

Statistical analysis for the quantitative data was processed using Mass Profiler Professional (MPP, version B.13.1) software (Agilent Technologies). Un-paired t-test and ANOVA with a multiple testing correction (Benjamini-Hochberg False Discovery Rate) were used to determine significant differences of the quantitative results of selected metabolites, with the criteria of frequency higher than 80 %, fold change higher than 1.5 and p values below 0.05 between the groups. The quantitative results were normalized by the creatinine concentrations which were determined using the creatinine assay kit purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

3. Results and Discussion

3.1. Self-administration

The number of methamphetamine infusions by lever pressing is shown in *Fig.* 2. On the ninth day, the results of daily 2 h methamphetamine self-administration sessions indicate that the number of lever pressing had steadily increased and showed a variation of less than 20 % during the last 3 days of the experiment. Urine (M2) was collected after 24 h from the final self-administration session to exclude methamphetamine itself as markers for methamphe-

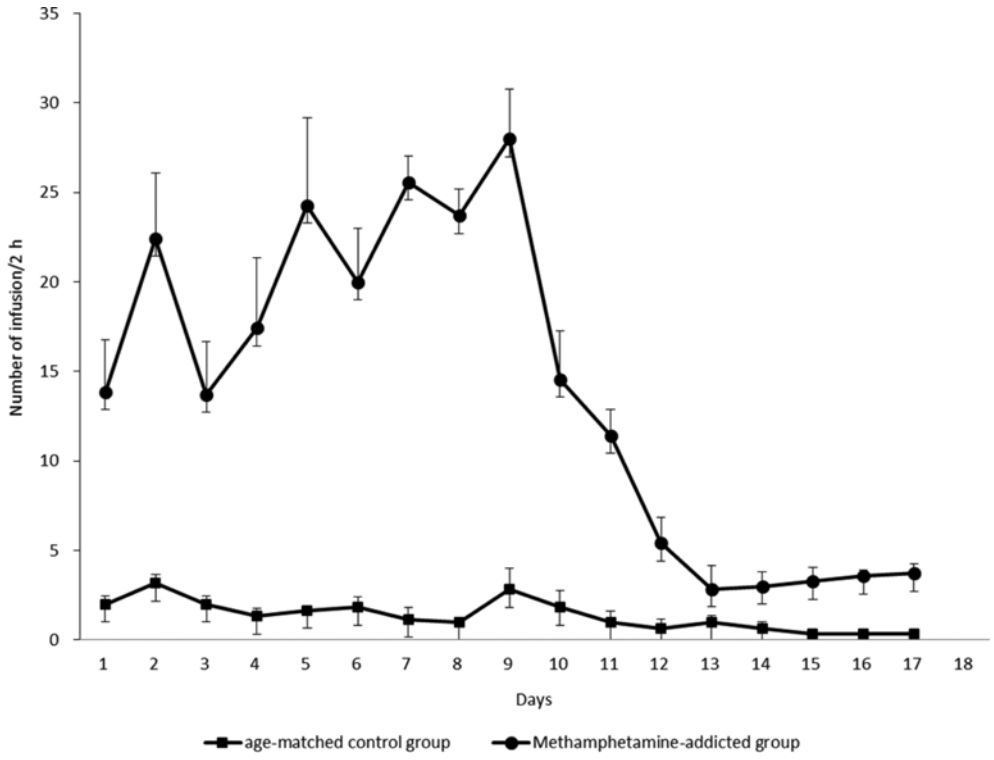

Fig. 2. Number of infusion during consecutive 2 h/day test session during the self-administration experiment (mean ± SEM, n = 6 for the age-matched control group, n = 7 for the methamphetamine-addicted group).

tamine addiction. The mean (± SEM) number of infusion on day 9 was 28.0 ± 2.8. Since then, extinction was performed and a variation of less than 20 % during the last 3 days (15-17 days) was shown. Urine (M3) was collected on day 18, on which the mean (± SEM) number of infusion was 3.7 ± 0.6. At the same time, the saline self-administration was conducted to aged-matched rats as controls, during which one rat died. Therefore, six rats for the age-matched control group and seven rats for the methamphetamine-addicted group were used for further experiments.

3.2. Metabolite analysis

The simultaneous quantification of a total of 187 metabolites was performed in the collected rat urine samples. Among the targeted metabolites, only 46 metabolites were (semi-) quantified and most of them were amino acids and biogenic amines (30 out of 41, *Table* 1). This is probably due to the higher endogenous concentrations of those metabolites than those of lipids in urine.[15]

Table 2 summarized that significantly changed metabolites among the experimental groups with p values and fold changes. Even though quantitative results were obtained in the urine samples, it was not considered as authentic concentrations of metabolites in urine because the calibrators used for quantification were not matrix-matched. Therefore, fold changes were used to describe significantly changed metabolites. *Fig.* 3 depicts a diagram of changes of metabolites between the age-matched control group and the methamphetamine-addicted group. The concentrations of spermidine and trans-4-hydroxyproline decreased with the same pattern between 'from C1 to C3' and 'from M1 to M3'. Those metabolites could be altered probably due to aging. Nevertheless, some metabolites, including aspartate, phenylethylamine, acetylcarnitine, asparagine, threonine, and taurine, were still significantly changed among the control groups, C1, C2 and C3, which might be caused by different environmental adaptation of the rats during the experimental period. Among the methamphetamine-addicted groups, M1, M2 and M3, the levels of lysine, acetylornithine and methioninesulfoxide were

Table 1. List of (semi-) quantified metabolites in urine samples

Metabolites	Metabolite group
Carnitine	Acylcarnitines
Tetradecanoylcarnitine	Acylcarnitines
Acetylcarnitine	Acylcarnitines
Butyrylcarnitine	Acylcarnitines
Alanine	Amino acids and biogenic amines
Arginine	Amino acids and biogenic amines
Asparagine	Amino acids and biogenic amines
Aspartate	Amino acids and biogenic amines
Citrulline	Amino acids and biogenic amines
Glutamine	Amino acids and biogenic amines
Glutamate	Amino acids and biogenic amines
Glycine	Amino acids and biogenic amines
Histidine	Amino acids and biogenic amines
Isoleucine	Amino acids and biogenic amines
Lysine	Amino acids and biogenic amines
Methionine	Amino acids and biogenic amines
Ornithine	Amino acids and biogenic amines
Phenylalanine	Amino acids and biogenic amines
Proline	Amino acids and biogenic amines
Serine	Amino acids and biogenic amines
Threonine	Amino acids and biogenic amines
Tyrosine	Amino acids and biogenic amines
Valine	Amino acids and biogenic amines
Acetylornithine	Amino acids and biogenic amines
cis-4-Hydroxyproline	Amino acids and biogenic amines
Dopamine	Amino acids and biogenic amines
Methioninesulfoxide	Amino acids and biogenic amines
Phenylethylamine	Amino acids and biogenic amines
Putrescine	Amino acids and biogenic amines
Serotonin	Amino acids and biogenic amines
Spermidine	Amino acids and biogenic amines
Spermine	Amino acids and biogenic amines
trans-4-Hydroxyproline	Amino acids and biogenic amines
Taurine	Amino acids and biogenic amines
lysoPC a C14:0	Lysophosphatidylcholines
PC aa C26:0	Phosphatidylcholines
PC aa C40:6	Phosphatidylcholines
PC aa C40:1	Phosphatidylcholines
PC aa C42:2	Phosphatidylcholines
PC ae C36:0	Phosphatidylcholines
PC ae C38:0	Phosphatidylcholines
PC ae C42:0	Phosphatidylcholines
PC ae C42:1	Phosphatidylcholines
PC ae C42:5	Phosphatidylcholines
PC ae C44:4	Phosphatidylcholines
Sum of Hexoses (including Glucose)	Monosaccharides

Table 2. List of significantly changed metabolites among the experimental groups

Comparing groups	Metabolites	P value	Fold change	Regulation
C1 vs. C2	Aspartate	2.53e-3	1.87	up
	Phenylethylamine	1.27e-2	1.57	up
C2 vs. C3	Acetylcarnitine	1.51e-5	-1.67	down
	Phenylethylamine	3.25e-2	-1.59	down
C1 vs. C3	Acetylcarnitine	5.06e-3	-1.92	down
	Asparagine	1.01e-6	-1.44	down
	Threonine	1.01e-6	-1.51	down
	Spermidine	1.90e-3	-1.96	down
	trans-4-Hydroxyproline	1.01e-6	-1.71	down
	Taurine	7.14e-3	1.54	up
C2 vs. M2	Acetylornithine	4.57e-2	-1.91	down
M1 vs. M2	Lysine	7.84e-3	-1.52	down
	Spermidine	7.01e-4	-1.63	down
M2 vs. M3	Acetylornithine	7.65e-4	2.84	up
M1 vs. M3	Acetylornithine	2.95e-2	1.93	up
	Methioninesulfoxide	9.79e-3	1.54	up
	Spermidine	4.25e-5	-1.78	down
	trans-4-Hydroxyproline	6.86e-5	-1.59	down

Lysophosphatidylcholine acyl, lysoPC a; Phosphatidylcholine diacyl, PC aa; Phosphatidylcholine acyl-alkyl, PC ae; Sphingomyeline, SM

P values were calculated using un-paired t-test and analysis of variance with a multiple testing correction (Benjamini-Hochberg False Discovery Rate).

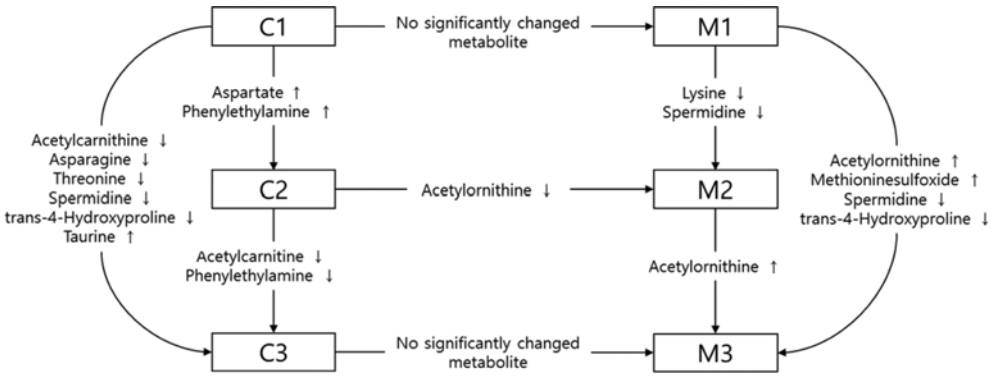

Fig. 3. Diagram of changes of metabolites between the age-matched control group and the methamphetamine-addicted group.

distinctively altered. Among them, the concentration of acetylornithine decreased in M2, compared with C1 ($p < 0.05$), C2 ($p < 0.05$) or M1 ($p = 0.07$) while that was recovered to the level in the control group (C3) following extinction (C3 vs. M3; $p = 0.54$) (*Fig.* 4). The concentrations of both dopamine and serotonin, the major neurotransmitters considered to be related with drug addiction, were not significantly changed among all the groups of samples.

Lysine deficiency was reported in patients with Parkinson's, hypothyroidism, kidney disease, asthma and depression. Methionine sulfoxide was suggested as a biomarker of oxidative stress in vivo.[16] However, any relation with methamphetamine addiction could not be decisive with the current results. Acetylornithine is a minor components of deproteinized blood plasma

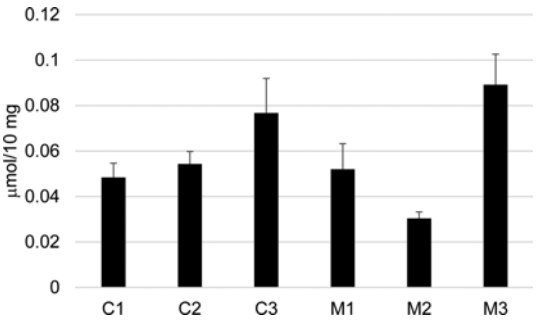

Fig. 4. Comparison of the acethylornithine levels (adjusted by creatinine concentrations, mean ± SEM, n = 6 for C1, C2 and C3, n = 7 for M1, M2 and M3).

of human blood and is an intermediate from glutamate to ornithine in arginine biosynthesis.[16] It was previously reported that higher acetylornithine concentrations were related to the chronic kidney disease.[17,18] A recent study on neurochemical metabolomics demonstrated that the chronic exposure of haloperidol, an antipsychotic drug, to mice caused to reduce the level of acetylornithine in brain.[19] However, no other previous studies were found on the relationship of acetylornithine with exposure to central nervous system acting drugs or other neurotoxic effects, which need to be further studied.

4. Conclusions

Metabolic alteration in rat urine by methamphetamine self-administration was investigated in the present study. The levels of lysine, acetylornithine and methioninesulfoxide were distinctively altered depending on the status of metheamphetamine addiction or extinction. In particular, the level of acetylornithine was reversely changed from addiction to extinction, for which further studies could be useful for biomarker discovery or mechanistic studies for methamphetamine addiction.

Acknowledgements

This research was supported by a grant from the Ministry of Food and Drug Safety (14182MFDS979) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014R1A1A1036222).

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