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Note

Metabolomic profiling of brain tissues of mice chronically exposed to heroin

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ABSTRACT

The chronic neurotoxicity of heroin on the nervous system is poorly understood. To address this issue, we comprehensively assessed the alteration of brain metabolomics caused by chronic heroin exposure and the withdrawal of heroin. Male C57BL/6J mice (n = 10) were given heroin (15 μmol/kg, i.p., twice a day) for 12 days while the withdrawal group received saline-treatment instead of heroin for the last two days. The control group received saline. We developed an UPLC-TOF/MS-based metabolomic approach to analyze the metabolites and carry out a metabolic pathway analysis in the brain. The major metabolites contributing to the discrimination were identified as amino acids, tricarboxylic-acid cycle intermediates, neurotransmitters, nucleotides and other compounds. A marked reduction in histidine and a slight but significant increase in phenylalanine and tryptophan were observed after heroin was withdrawn while the increased level of catecholamines was restored to baseline. Interestingly, N-acetylserotonin - a precursor of melatonin - was increased with the withdrawal of heroin while melatonin was markedly reduced along with the sub-chronic exposure to heroin. This shows that heroin disrupts not only the energy metabolism but also the biosynthesis of both catecholamines and melatonin in the mouse brain. Therefore, these substances are candidate biomarkers for chronic heroin-abuse.

1. Introduction

Heroin is the most widely abused opioid in the world [1]. It can lead to addiction quickly and does great harm to individuals, both physiologically and psychologically [2]. It can be rapidly metabolized to morphine and other metabolites, which can bind to opioid receptors in the brain [3]. Clearly, drug-induced stable changes in the brain at the molecular level would produce addictive behavior. Repeated heroin use changes the physical structure and physiology of the brain [4], creating long-term imbalances in neuronal and hormonal systems which are not easily reversed [5,6].

Metabolomics aims to extract, separate and analyze the totality of small molecules present in any biological system (such as biofluids and tissues) or any specific physiological state in an effective and reproducible way [7]. It has been widely applied in toxicological studies, especially when investigating the action mechanism of toxicity [8,9]. Furthermore, because heroin can be rapidly metabolized in vivo, heroin itself cannot be used as the target analyte to identify heroin abuse in practice. Therefore, we use endogenous compounds as metabolic markers to identify heroin abuse and this is an alternative approach, which can also reveal the underlying mechanisms.

Previous metabolomics studies have shown metabolic alterations in rats exposed to heroin using blood and urine samples [2]. However, the effect of heroin on the brain metabolome has not been clarified and the sub-chronic neurotoxicity of heroin at low levels in the brain is poorly understood. The purpose of this study was to identify the potential brain biomarkers and the molecular neurotoxicity mechanisms in mice exposed to heroin and following heroin withdrawal.

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2. Materials and methods

2.1. Chemicals and reagents

Heroin hydrochloride was synthesized by acetylation of the two-hydroxyl groups of morphine with acetic anhydride in our laboratory [10]. All other reagents were of the highest grade commercially available.

2.2. Animal treatment and sample collection

Male C57BL/6J mice (20–25 g) were purchased from CLEA Japan, Inc (Tokyo, Japan). The mice were housed in a temperature-controlled environment (20 ± 4 °C), with a 12 h light-dark cycle (lights on at 7: 00) and a relative humidity of 50 ± 20%, with free access to food and water. After adaptation to standard laboratory conditions for 1 week, the mice were randomly allocated to 3 groups (n = 10/group): a heroin-treated group, a heroin-withdrawal group and a control group. All animal experiments were conducted with the approval of the Institutional Animal Care and Experiment Committee of Kyushu University.

Heroin hydrochloride was dissolved in saline on the day of use and all mice were weighed every day prior to administration. The mice in the heroin-treated group received an i.p. injection of heroin (15 μmol/kg) twice a day (9 am and 5 pm, respectively) for 12 consecutive days. The dose used was chosen according to the published studies [11,12]. The mice in the heroin-withdrawal group received an i.p. injection of heroin (15 μmol/kg) twice a day for 10 consecutive days followed by withdrawal of heroin for 2 days. The mice in the control group received an i.p. injection of saline for 12 days. Then, the mice were anesthetized by CO_2 inhalation and their brains were rapidly removed, and rinsed with saline. Finally, the brain tissues were transferred to 1.5 ml polypropylene tubes, snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

A liquid–liquid extraction method was used for metabolite extraction from each brain tissue sample [13]. The residue was finally reconstituted in 100 μl CH_3CN/H_2O (1:1, v/v), and an aliquot (10 μl) of the solution was subjected to analysis by UPLC-TOF/MS (see the Supplemental Methods S1 for the detailed conditions).

2.3. Data processing and metabolite identification

Data processing was performed using MassLynx v4.1 and MarkerLynx XS v4.1 software (Waters Co., Milford, MA). To compare metabolomic profiles between the control and heroin-treated group or between the control and heroin-withdrawal group, the data were then analyzed by an orthogonal partial least squares-discriminant analysis (OPLS-DA) and S-plot method using MarkerLynx XS v4.1 software [14,15]. (see the Supplemental Methods S2 for the detailed conditions).

3. Results and discussion

After heroin administration, the mice became excited, nervous and very sensitive to disturbances. Subsequently, after being excited for approximately 1 h, the mice gradually became calm. In addition, there was a reduction in body weight immediately after heroin administration (see Supplemental Fig. S1).

We subsequently used a multivariate analysis method with UPLC-MS data to make metabolite variations prominent and to identify the metabolites responsible for such variations. The corresponding score plots from the supervised OPLS-DA showed a clear difference between the two groups (Fig. 1). The S-plot, which was derived from the OPLS-DA, was used to select potential biomarkers and visualize metabolomics changes. The farthest red dots were the most likely potential biomarkers because of their high contributions and correlations. A list of the identified biomarkers is given in Table 1. The intensities of the peaks obtained from MarkerLynx were used to calculate the relative concentrations of the biomarkers.

Some selected biomarkers were further investigated. These reflect the metabolic responses of the mice to heroin exposure and can reveal potential markers of metabolic perturbation and provide data for exploring the underlying mechanisms of action. Citrate is a crucial intermediate of the tricarboxylic acid (TCA) cycle [2]. Elevation of citrate indicates up-regulation of the TCA cycle. It has been reported that loss of appetite is one of the symptoms of heroin addiction [16]. On the other hand, in response to heroin administration, mice consumed more energy source materials (Table 1). Since it is known that body weight loss is also associated with energy consumption [2,17], the heroin-induced effect was probably associated with similar mechanisms. Nucleotide monophosphates, which are metabolites of nucleotide triphosphates, were increased significantly in the brain following heroin treatment. It is generally believed that nucleotide metabolomics can provide much energy, resulting in increased production of nucleotide monophosphates [18]. Glutamate is an important excitatory neurotransmitter that plays the principal role in central nervous system (CNS) [19]. Elevation of glutamate suggested excitation of the animals by chronic heroin exposure.

Furthermore, heroin can lead to autonomic nervous-system dysfunction and neurotoxicity. Catecholamines in the brain are thought to play a role in mood regulation [20]. In our study, tyrosine was reduced after heroin administration. In CNS, there are many kinds of important neurotransmitters related to the metabolism of tyrosine; e.g. dopamine, adrenaline and noradrenaline. The metabolic pathway of tyrosine in the CNS is shown in Supplemental Fig. S2. These peripheral metabolites were identified as potential surrogate markers characterizing the metabolic effect of heroin on CNS function. Although both dopamine and adrenaline were elevated by chronic heroin exposure, the effect on the level of adrenaline was far lower than that for dopamine, whereas the level of these catecholamines returned to baseline after the withdrawal of heroin for 2 days. The significant increase in dopamine might be characteristic of heroin-induced toxicity in the brain [21]. Further studies are necessary to elucidate the mechanism underlying the complex modulation of catecholamines by heroin-exposure. These catecholamines may be indirect biomarkers and can not only be used to understand the whole system, but also reveal new insights into the mechanism of toxicity. Interestingly, phenylalanine, a precursor of catecholamine showed a slight but significant increase in the withdrawal group. Furthermore, heroin produces an elevation of histamine while a marked decrease in histidine was observed after heroin withdrawal. This agrees with the report that morphine increases the release of histamine from mast-cells [22]. However, the mechanism for the reduction in histidine following heroine withdrawal remains to be elucidated. Tryptophan and N-acetylserotonin, precursors of melatonin, were increased by heroin-withdrawal while melatonin was markedly reduced by sub-chronic exposure to heroin. Melatonin supplementation is known to be associated with the syndrome elicited by heroin withdrawal [23]. Therefore, a reduction of melatonin is a possible biomarker of heroin abuse while an increase in tryptophan and N-acetylserotonin are possible biomarkers of heroin withdrawal. Further studies are necessary to clarify the effect on the levels of mRNA and protein expression of genes related to these pathways.